ANTIVIRAL DEFENCE AND ANTIBACTERIAL PROTEINS IN THE SHORE CRAB CARCINUS MAENAS

Denni Schnapp

A Thesis Submitted for the Degree of PhD
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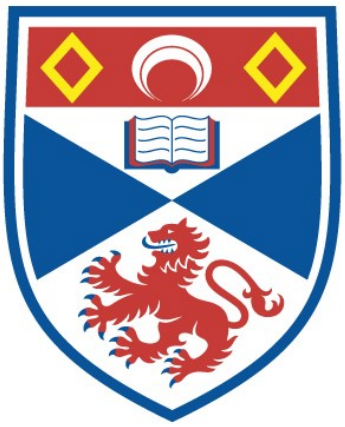

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ANTIVIRAL DEFENCE AND ANTIBACTERIAL PROTEINS IN THE SHORE CRAB, *CARCINUS MAENAS*

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SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MAY 1996

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To Mutti and John

Declarations

(i) I, Denni Schnapp, hereby certify that this thesis, which is approximately 48, 500 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Abstract

The defence reactions of the shore crab, *Carcinus maenas*, to a range of viruses were investigated *in vivo* and *in vitro*. *In vivo* studies with injected bacteriophages showed that *C. maenas* is capable of discriminating between different bacteriophages and actively removes certain phages from the haemocoele. Rapid initial clearance of the coliphage T_2 was followed by slower removal and the phage persisted in the circulation for at least two weeks. The phage was sequestered to the hepatopancreas where it persisted for at least 72 h. Haemocyte counts remained unchanged upon injection of T_2. With respect to prophenoloxidase activation, of the viruses tested only the *Chlorella* phage PBCV-1 was found to activate haemolymph prophenoloxidase at concentrations above 10^7 particles ml^{-1}. This indicates that *C. maenas* may respond to high concentrations of viruses *in vitro*. However, neutralization assays failed to reveal inactivation of viruses in HLS, plasma, or extracts of the hepatopancreas, gut, gill or heart, although some activity against an insect baculovirus and parainfluenza virus was detected in digestive juice.

At least four antibacterial proteins, of ca. 6.5 kDa, 11.6 kDa, 20 kDa or 25 kDa, are present in *C. maenas* haemocytes. One, a 6.5 kDa peptide with activity against Gram positive and Gram negative bacteria, was purified. The N-terminal 30 amino acids of this peptide share over 60% sequence identity with bovine Bac 7, a mammalian cathelicidin. This 6.5 kDa peptide in *C. maenas* is the first antimicrobial peptide described from the Crustacea. Because the sequence of the pre-propeptide is as yet unavailable, it is not known whether or not it can be included among the cathelicidins. It has not been established whether or not the *C. maenas* 6.5 kDa peptide has antiviral activity.

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Abbreviations

ACN	Acetonitrile (methyl cyanide)
AcNPV	*Autographa californica* nuclear polyhedrosis virus
ANOVA	Analysis of variance
APS	Ammonium persulphate
AS	Ammonium sulphate
Bac5	Bactenecin 5 (bovine)
Bac7	Bactenecin 7 (bovine)
Bf	bluegill fibroblast cell line
βGBP	β-1,3 glucans binding protein
BmNPV	*Bombyx mori* nuclear polyhedrosis virus
BSA	Bovine serum albumin
CAC I-III	Sodium cocodylate buffers I-III
CM-Sephadex	Carboxymethyl Sephadex
CS	*Carcinus* saline
cfu	Colony forming units
CGSC	*E. coli* Genetic Stock Collection (Yale University, USA)
DW	Deionised (MilliQ) water
EDTA	Ethylenediaminetetraacetic acid disodium salt
FCS	Fetal calf serum
G-MEM	Glasgow minimum essential medium
HIV	Human immunodeficiency virus
HLS	Haemocyte lysate supernatant
HPLC	High performance liquid chromatography
HSV I	Herpes simplex virus I
IHHN	Infectious hepatopancreatic and hemopoietic necrosis virus
IPN	Infectious pancreatic necrosis virus (fish pathogen)

LB	Luria Bertani (formula for nutrient broth)
L-dopa	L-Di-hydroxyphenylanaline
LPS	Lipopolysaccharide
NPV	Nuclear polyhedrosis virus
MA	Marine anticoagulant
MSM	Marine salt magnesium buffer
NCIMB	National Collection of Industrial and Marine Bacteria
NMWL	Nominal molecular weight limit
PAGE	Polyacrylamide gel electrophoresis
PB	Phosphate buffer
PBS	Phosphate buffered saline
PdNPV	*Penaeus duorarum* nuclear polyhedrosis virus
pfu	Plaque forming units
PmNPV	*Penaeus monodon* nuclear polyhedrosis virus
PO	Phenoloxidase
PTU	Phenylthiourea
RP-HPLC	Reverse phase high performance liquid chromatography
SDS	Sodium dodecyl sulphate
SE	Standard error of the mean
Sf9	*Spodoptera frugiperda* ovarian cell line (ATTC CRL-1711)
SM	Salt magnesium buffer
$TCID_{50}$	50 % tissue culture infective dose
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
VSV	Vesicular stomatitis virus

1. General introduction

1.1 IMPORTANT PATHOGENS OF CRUSTACEANS

Introduction: The economical importance of crustaceans

Crustaceans are a diverse and important group of mainly marine invertebrates. They contribute to world fisheries both as part of the aquatic foodchain and as commercially exploited stocks (Brock & Lightner, 1990). Many important fish stocks depend on copepod naupilii as primary food source for their juvenile stages (e.g. Macer, 1991, Cochraine & Hutchings, 1995), and krill (*Euphausia superba*) is at the base of the antarctic food chain. Crustaceans themselves accounted for 4.5% of world fishery landings in 1989 (FAO, 1991). The most important species group is shrimp (= prawns), which comprised 55.8% of total crustacean production, whereas crabs and lobsters constituted about 30% (FAO, 1991) (Table 1.1.1). The value of certain species considerably exceeds that of finfish and crustaceans therefore contribute disproportionally to the fisheries of some countries. For example, penaeid prawns are almost 10 times as valuable as finfish by weight, and in some tropical countries the value of shrimp trawling almost matches that of the entire remaining fishery (Pauly, 1987). With overfishing and excessive bycatches leading to trawling restrictions (Pauly, 1987), the farming of penaeid shrimp has expanded during the past decade (Rosenberry, 1991; Fulks & Main, 1992). The high export value and rapid growth of tropical penaeids has lead to the development of intensive farming techniques which brought about a near doubling of the global output of shrimp aquaculture during the 1980s, from 300, 000 metric tons (MT) to 565, 000 MT (Rosenberry, 1991). Production at present is 712 000 MT (Dr. J. Lotz, Gulf Coast Research Laboratory, Ocean Springs, Massachusets, USA, pers. com.), which represents a further 26% increase. Cultured shrimp presently accounts for ca. 28% of the worldwide production of penaeids (Lotz pers. com.).

Table 1.1.1 Economically important species of crustaceans, listed in order of landings (metric tons) in 1989 (FAO, 1991)

Species	Main producers	Economic importance	References
Euphausia superba (antarctic krill)	Former USSR, Japan		FAO (1991)
Acetes japonicus (Akiami paste shrimp)	China, Korea Rep.		FAO (1991)
**Penaeus orientalis (chinensis)* (fleshy prawn)	China, South Korea	Also important farmed species (ca. 80% of farmed shrimp in China in 1992).	FAO (1991); Fulks & Main (1992)
Pandalus borealis (northern prawn)	N. Europe		FAO (1991)
Chionectes spp. (Pacific snow crab)	Canada		FAO (1991)
** Penaeus merguiensis* (Banana prawn)	Indonesia, Thailand	Important farmed species in Indonesia, Vietnam, Thailand and the Philippines	FAO (1991); Dore & Frimodt (1987)
** P. occidentalis* (western white prawn)	Ecuador, Colombia		FAO (1991)
Trachypenaeus cuvirostris (cocktail shrimp)	China		FAO (1991)
** P. aztecus* (northern brown prawn)	USA	Minor species in aquaculture in Americas	FAO (1991); Fulks & Main (1992)
Homarus americanus (American lobster)	USA	Experimental aquaculture, holding ponds	FAO (1991); Sindermann (1988a)

* *P. monodon* (giant tiger prawn)	Indonesia, Thailand	Most important cultured species worldwide.	FAO (1991); Fulks & Main (1992)
Callinectes sapidus (blue crab)	USA	Experimental culture, shedding tanks in USA. In Japan 7% of crust. farm production	FAO (1991); Sindermann (1988b); (Momoyama, 1992)
Nephrops norvegicus (Norway lobster)	N. Europe	Scotland main producer (32%).	FAO (1991)
Portunes trituberculatus (Gazami crab)	Korea Rep.		FAO (1991)
P. setiferus (North. white prawn)	USA		FAO (1991)
Panurus argus (spiny lobster)	Caribbean, Cuba		FAO (1991)
Procambarus clarkii (red swamp crayfish)	USA		FAO (1991)
Portunes pelagicans (sand crab)	Thailand		FAO (1991)
Cancer pagurus (edible crab)	N. Europe	UK, France biggest producers	FAO (1991)
* *Macrobrachium rosenbergii* (giant river prawn)	SE Asia (Thailand, Brunei Darussalam)	Important farmed species in SE Asia, some culture in Americas, Jamaica	Brock (1988); FAO (1991)
P. japonicus (Kuruma shrimp)	Japan, South Korea, Taiwan	95% of farmed shrimp produced in Japan , 30% in Taiwan in 1991	FAO (1991); Fulks & Main (1992);
P. vannamei	Colombia, Ecuador, Hawaii, Honduras, Peru	Small fishery, but 80% of cultured shrimp in Ecuador, 17% worldwide	Rosenberry (1991) Fulks & Main (1992)

* Important aquaculture species

Commercially valuable crustaceans suffer from a range of debilitating diseases. Non-infectious diseases are caused by environmentally adverse conditions, rather than pathogens. Their effect on crustaceans is often difficult to distinguish from that of infectious diseases, not least because environmental stress factors lower the defence capabilities of the host (Smith & Johnston, 1992; Smith *et al.*, 1995), thus allowing invasion by opportunistic pathogens (Couch, 1974; 1976; Lightner, 1993). In wild populations, the occurence of epizootics triggered by environmental stress has been a major concern (Couch, 1988), but direct effects of environmental stress have also been observed, e.g. anoxia in Baltic lobsters during the bloom of the alga, *Chrysochromulina polylepis* in the Kattegat and Skagerrak in 1988 (Lindahl & Dahl, 1990). The true impact of disease on wild populations is unknown (Stewart, 1993), but its impact on aquaculture is of increasing concern (Fulks & Main, 1992). Non-infectious diseases are caused mainly by inadequate diet and husbandry and are increasingly being eliminated by improvements in culture techniques. These improvements may also lead to increased resistance against pathogens. However, infectious diseases, especially viral diseases, continue to cause losses. Work presented in this thesis addresses host defence against viral and bacterial infections in marine crustaceans. Therefore, some of the important pathogens of crustaceans will be regarded, followed by an account of the crustacean immune response.

Rickettsial, bacterial, fungal and protozoan pathogens

Crustaceans share their environment with a large number of microorganisms, most of which are free-living, but can cause opportunistic and occasionally severe infections (Stewart, 1993). Table 1.1.2 lists some of the most important rickettsial, bacterial, fungal and protozoan pathogens. Crustaceans are also affected by other parasites, such as helminths and parasitic crustaceans, but these do not appear to have a significant impact on populations (Sindermann, 1988a; Stewart, 1993).

Table 1.1.2. Some important rickettsial, bacterial, fungal and protozoan pathogens of cultured crustaceans

Pathogen	Species affected	Range	Effects	References
Rickettsia:				
Rickettsial infection of shrimp	*Penaeus marginatus, P. merguiensis, P. monodon, P. vannamei*	Hawaii, Malaysia, Singapore	Slow growth, occasional mortalities. Not considered serious pathogens of cultured shrimp	Brock *et al.* (1985) Brock (1988 b)
Bacteria:				
Aerococcus viridans (Gram positive)	*Homarus americanus, H.vulgaris*	Canada (east coast), Europe, USA (east and west coast)	Invariably lethal. Important pathogen affecting lobster fishery	Sniezko & Taylor (1947) Stewart (1993)
Vibrio spp., *V. anguillarum* (Gram negative)	*Penaeus* spp., *Callinectes sapidus, Cancer* spp., *Carcinus* spp. *Homarus* spp.	ubiquitous	Opportunistic, can cause severe mortalities in stressed animals. Important pathogen of shrimp aquaculture	Fulks & Main (1992) Lightner (1988 a) Rosemark & Fisher (1988)
Vibrio, Aeromonas, Spirillum, Flavobacterium spp. (mostly Gram negative)	*Penaeus* spp., *Callinectes sapidus, Cancer* spp., *Carcinus* spp.*H.omarus* spp.	ubiquitous	Produce extracellular enzymes breaking down cuticle (shell disease) in stressed animals	Cook & Lofton (1973) Lightner (1988 b)

Fungi:				
Lagenidium callinectes, *Sirolpidium* spp., *Haliphtoros* spp.	*Penaeus* spp., *C. sapidus (Lagenidium callinectes)*	ubiquitous	Egg and larval mycosis of penaeid shrimp or *C. sapidus*. Can lead to severe losses	Lightner (1988 c) Fulks & Main (1992)
Fusarium spp.	all *Penaeus* spp., susceptibility differs between species, *Homarus* spp.	ubiquitous	Stress-related outbreaks, causes "black gill disease" in shrimp; black spot disease in lobsters	Lightner (1988 d) Lightner & Fontaine (1975)
Aphanomyces astaci	*Astacus* spp., *Pacifastacus* spp.	SE Asia, Europe	Decimated economically valuable *Astacus* spp. in Asia and Europe; *Pacifastacus* more resistant	Stewart (1993)
Protozoa:				
Microsporida (*Agmasoma, Ameson, Pleistophora* spp.)	all *Penaeus* spp., *C. sapidus*	ubiquitous	"Cotton shrimp disease" (infected muscle turns opaque). Most severe protozoan pathogen in shrimp aquaculture but not a major problem as it requires an intermediate host	Lightner (1988 e) Fulks & Main (1992) Stewart (1993)
Hematidinium-like dinoflagellate	*Chionoecetes opilio, C. bairdi* (Alaskan tanner crabs)	Arctic	"Bitter crab syndrome", up to 35% prevalence, hence significant economic losses in local fishery	Meyer *et al.* (1987)

Many of the pathogens listed in Table 1.1.2, such as the bacteria, *Aerococcus viridans* and *Vibrio* spp., the fungi *Aphanomyces astaci*, *Fusarium* spp. and *Lagenidium* spp., or microsporidian Protozoa, have caused significant losses. Others, such as the rickettsia, have not caused significant problems in cultured populations, but are likely to be of importance in the wild (Fulks & Main, 1992) (Table 1.1.2).

The best known crustacean disease is Gaffkaemia (Snieszko & Taylor, 1947), a bacterial disease of American and European lobsters (*Homarus americanus* and *H. vulgaris*, respectively). The term derives from the former name (*Gaffkya homari*) of the pathogen *Aerococcus viridans* which causes the disease. *A. viridans* is a Gram positive free-living bacterium that enters the animals through wounds (Sindermann, 1988a; Stewart, 1993). It is resistant to antibacterial factors in the haemolymph, and although it is phagocytosed, it is not killed within the haemocytes (Cornick & Steward, 1968). As few as 10 viable bacteria per kg of bodyweight can mount a lethal infection (Stewart, 1993). Gaffkaemia is a primary disease of lobsters, i.e. it causes lethal infection even in animals not suffering from environmentally adverse conditions, which may cause outbreaks of opportunistic pathogens (Stewart, 1993). However, most bacterial diseases of crustaceans are caused by opportunistic (secondary) pathogens (Table 1.1.2). A potentially fatal bacterial disease which affects many species of crustaceans is vibriosis or Gram-negative septicaemia, caused by bacteria of the genus *Vibrio* (Lightner, 1988a; Rosemark & Fisher, 1988). Vibriosis is a major contributor to losses in shrimp aquaculture. Recent specific data of its importance are not available, but in 1984 it accounted for 44% of disease-related losses in cultured *P. japonicus* in Japan (Sano & Fukuda, 1987). *Vibrio* spp. and a number of other genera of chitinolytic, mainly Gram negative, marine bacteria can also break down the carapace of crustaceans upon penetration of the epicuticle (Cook & Lofton, 1973) and cause shell disease, often followed by secondary infections (Lightner, 1988b). Opportunistic bacterial diseases are a major concern in

wild populations exposed to environmental pollution (Couch, 1988). In aquaculture, they can generally be prevented by good husbandry (Lightner, 1988a; Rosemark & Fisher, 1988). Antibiotics and antibacterial agents are often effective in treating crustaceans suffering from bacterial infections. In addition, several authors have claimed that "vaccination" of lobsters (Stewart & Zwicker, 1974) or penaeids (Giorgetti, 1990; Itami & Takakashi, 1991; Larramore, 1992) may be successful in preventing outbreaks of bacterial infections.

One of the oldest recognized crustacean diseases is European crayfish plague, caused by a fungus, *Aphanomyces astaci* . It was identified in 1860 and has led to the decline of economically important freshwater crayfish of the genus *Astacus* in both Europe and Asia (Stewart, 1993). This fungal parasite is carried by several genera of crayfish and may have been introduced into Europe by more resistant exotic species, such as *Pacifastacus astacus* (Stewart, 1993). It forms a free-swimming zoospore that settles on the crayfish cuticle and breaks down the epicuticle and carapace by excreting lipases and chitinases (Unestam, 1975). Fungal hyphae become melanized, but in *Astacus astacus*, melanization is weaker than in other species of crayfish, and it is thought that this relatively low capacity for melanization is the factor responsible for the pathogenicity of the fungus against this genus (Unestam, 1975). Again, most fungal pathogens of crustaceans appear to be opportunistic (Table 1.1.2). Black gill disease of shrimp (Lightner, 1988c) and black spot disease of lobsters are caused by *Fusarium* spp. (Lightner & Fontaine, 1975), which are free-living but may cause infections after penetration through injury (Lightner, 1988c; Lightner & Fontaine, 1975). Species of penaeids differ in their susceptibility to *Fusarium* spp. (Lightner, 1988c; Fulks & Main, 1992), which may indicate differences in defence capacities of the hosts. Most opportunistic fungal infections tend to affect eggs and larvae, rather than unstressed postlarvae or adults (Lightner, 1988c). The fungus, *Lagenidium callinectes,* affects the eggs and early larval stages of several species of crabs (Stewart, 1993), as well as of penaeid shrimp (Lightner,

1988c; Fulks & Main, 1992). In addition to *L. callinectes*, the main causes for larval mycosis in penaeids are the fungi *Siroplidium* spp. and *Haliphtoros* spp (Lightner, 1988c; Fulks & Main, 1992). Larval mycosis can cause severe losses, but is treatable with antifungals such as Treflan or malachite green (Fulks & Main, 1992). Fungal diseases of adult crustaceans are difficult to treat (Lightner, 1988c).

Unlike any of the pathogens described so far, several species of protozoan Microspora (*Agmasoma, Ameson, Pleistophora* spp.) are obligate intracellular parasites of crustaceans (Lightner, 1988e, 1993; Fulks & Main, 1992; Stewart, 1993). These protozoans are known to be ubiquitous in wild populations (Lightner, 1988e), and cause "cotton shrimp syndrome", which can also affect crabs (Overstreet, 1988, Stewart, 1993). However, they appear to require an intermediate host in their life cycle and have therefore not caused major infections in shrimp aquaculture (Fulks & Main, 1992). Other protozoans which occur in crustaceans are rarely pathogenic (Fulks & Main, 1992). An exception is a *Hematidinium*-like dinoflagellate which affects tanner crabs (*Chionoecetes opilio* and *C. bairdi*) and has caused damage to the fishery of these species in Alaska (Meyers *et al*, 1987).

In conclusion, infestations by most rickettsial, bacterial, fungal and protozoal pathogens (Table 1.1.2) are opportunistic and tend to be prevalent under stressful conditions or in already diseased animals. Important exceptions are the bacterium *A. viridans*, affecting *Homarus* spp., the fungus *A. astaci*, affecting *Astacus* spp., and several protozoan Microspora. Infection by *A. viridans* or *A. astaci* of crustaceans other than their primary hosts does not lead to disease, although these organisms are capable of growing in these species (Stewart, 1993; Unestam, 1975). This indicates that crustaceans generally have an efficient defence against infection, but the capacity for host defence may differ between species (Stewart, 1993).

Viral diseases of crustaceans

Viruses are abundant in the marine environment. Up to 2.7×10^7 particles ml^{-1} have been identified in surface waters (Børsheim *et al.*, 1989). Most of these affect bacteria or phytoplankton (Suttle *et al.*, 1991). Viral predation on bacteria alone can reduce mesozooplankton production by 5-15%, thus indirectly affecting levels of exploitable marine resources (Murray & Eldridge, 1994). However, unlike most of the pathogens described so far, viruses are obligate parasites which replicate intracellularly and tend to remain in close association with their hosts. It is therefore unlikely that viruses present in the water column cause opportunistic infections in crustaceans.

Many, and possibly all, invertebrate phyla are affected by viruses. The main groups of invertebrate viruses are listed in Table 1.1.3. The nature of the viral genome has seldom been determined, hence the classification of most invertebrate viruses remains tentative. In addition to the groups listed in Table 1.1.3, uncharacterized viruses have been described from protozoans, platyhelminths, nematodes, annelids and chelicerates (Tinsley & Harrap, 1978). Recently, advances have been made in the characterisation of arthropod viruses, helped by improvements in molecular biology and driven by the quest for new pest control agents (Miller, 1995) and the need to develop diagnostic tools for viral diseases of commercial species (Lightner *et al.*, 1992). Among arthropod viruses, the most prominent group are baculoviruses, of which several hundred have been isolated mainly from Lepidoptera and Hymenoptera (Hull *et al.*, 1989). Baculoviruses are also the largest group of viruses known from decapod crustaceans (Johnson & Lightner, 1988 and see below). They, the entomopoxviruses and the polydnaviruses are the only viral groups which are restricted to the invertebrates. In addition,

Table 1.1.3 Groups of invertebrate viruses and host groups

Virus group	Hosts	Example	Nucleic acid	Characteristics	Comment	References
Adenoviridae	Cnidaria? Porifera?	*Hydra vulgaris* virus ?, *Verongia cavernicola* virus?	linear dsDNA	Naked, icosahedral, non-occluded, ca. 50-70 nm	Greek "adenos"= gland	Bonnefoy *et al.* (1972); Vacelet & Gallissian (1978)
Baculoviridae:			circular dsDNA	Arthropod viruses, rod-shaped, nucleic	"Baculum" = rod	Wildy (1971)
Granulosis virus	Lepidoptera			Granulosis virus: one virion per occlusion body	Often parasitoid-transmitted, marketed for pest-control.	Tanada & Hess (1991); Crook (1994)
single nuclear polyhydrosis virus, (SNPV)	Insecta, Crustacea	PvSNPV, PmSNPV of shrimp (Table 1.2.4)		SNPV: one virion per envelope, many per occlusion body, where present	SMPV include important shrimp pathogens (Table 1.2.4)	Adams & McClintock (1991); Couch (1991);
Multiple nuclear polyhydrosis virus (MNPV)	Lepidoptera	AcMNPV (*Autographa californica*)		MNPV: several viria per envelope, ditto.	AcMNPV used as gene expression system and in insect pest control.	Huger & Krieg (1991); Rohrmann (1994); Miller (1995)
Birnaviridae	Mollusca, Insecta, Rotifera, Crustacea	*Tellina* virus, *Drosophila* X- virus	dsRNA 2 segments	Naked, icosahedral	"Bi"-segmented "RNA" viruses. IPN of fish may infect aquatic invertebrates	Dobos *et al.* (1979); Comps *et al.* (1991); Becht (1994)
Bunyaviridae	Insecta, Crustacea	Arboviruses, S virus; S_2 virus (Table 1.1.4)	- ssRNA segments	Enveloped ovoid- filamentous, cytoplasmic	From type: "Bunyamwera", includes many arthropod-borne vertebr. viruses	Nathanson & Gonzales-Scareno (1994)
Entomopox-viridae	Insecta		linear dsDNA	Spherical-ovoid or "brick"-shaped, enveloped, occluded	"Insect pox viruses", similar structure to poxviruses but no common antigens	Vago (1963); Goodwin *et al.* (1991)

Herpesviridae	Mollusca ?, Crustacea ?	Cheasapeake Bay virus (Table 1.2.4)	linear dsDNA	Enveloped, icosahedral, non-occluded	Mostly vertebrate viruses, incl. human HSV I, HSV II	Farley *et al.* (1972) Tinsley & Harrap (1978)
Iridoviridae	Insecta, Crustacea, Annelida, Mollusca	*Tipula* iridovirus (first described iridovirus)	linear dsDNA	Naked, icosahedral, cytoplasmic	Greek "iridos"= irridescent. Infect all vertebrate groups. Incl. fish lymphocystis	Xeros (1954); Hull *et al.* (1989)
Parvoviridae	Insecta , Crustacea	IHHN (Table 1.2.4)	ssDNA	Naked, icosahedral, small (20-26 nm)	Lat. "parvus"= small. Incl. Lepidopteran densoviruses, but mainly vertebrate viruses	Pattison (1994); Hull *et al.* (1989)
Picornaviridae	Insecta, Crustacea?	*Drosophila* C-virus	+ ssRNA	Naked, icosahedral, small (ca. 30 nm), cytoplasmic	Old Span. "pico"= micro "RNA" viruses	Sotti (1994); Hull *et al.* (1989)
Polydnaviridae	Insecta (Hymenoptera)	*Campoletes sonorensis* polydnavirus	supercoiled DNA segments		"Polydisperse DNA viruses". Symbiotic in parasitoid wasps, surpress egg-encapsulation by host larvae	Edson *et al.* (1981); deBuron & Beckage (1992) Stoltz (1994)
Reoviridae	Crustacea, Mollusca, Arachnida, Acoelomata		dsRNA segments	Usually non-occluded, naked, icosahedral, cytoplasmic	Includes cytoplasmic polyhydrosis viruses of insects	Devauchelle & Vago (1971); Morel (1975); Mohktar-Maamouri *et al.* (1976); Belloncik (1994)
Rhabdoviridae	Insecta, Crustacea	*Drosophila* σ virus	- ssRNA	Non-occluded, naked, rod-shaped	Greek "rhabdos"= rod.	Hull *et al.* (1989); Teninges (1994)
Togaviridae	Mollusca? Crustacea	Shrimp LOVV (Table 1.1.5)	+ ssRNA	Non-occluded, enveloped rods.	Lat. "toga"= cloak	Farley, 1978; Hull *et al.* (1989); Spann *et al.* (1995)

ds = double stranded, ss = single stranded, - ssRNA = antisense single stranded RNA (requires reverse transcripase), + ssRNA = sense single stranded RNA

diseases such as dengue fever, yellow fever or encephalitis are caused by arthropod-bone viruses ("arboviruses", a collective term for viruses from several taxonomic groups), which are transmitted to vertebrates by insect or ticks in which the viruses also replicate. Shared features between the viruses of a group which infects many species may indicate common ancestry, and it is likely that the host specificity of viruses is a result of host-virus co-evolution (Morse, 1994 and see below).

The first virus to be identified in a marine invertebrate was found in the swimming crab, *Macropipus depurator* (Vago, 1966). This virus was later included among the Reoviridae (P virus; Bonami, 1980). From the 1970s onwards several more viruses were identified in crabs (Table 1.1.4), usually in animals which displayed signs of disease, namely paralysis (Vago, 1966; Bang, 1971; Johnson, 1977) or impaired blood clotting (Bang, 1971; Johnson, 1976). Subsequently, it was found that experimental infections with the newly isolated viruses often lead to proliferation of latent viruses (Johnston, 1977; Bonami, 1980; Mari 1987). Thus, Johnston (1977) found that a reo-like virus infection in the blue crab, *Callinectes sapidus*, always co-occured with a rhabdo-like virus infection. Similarly, Bonami (1980) observed a co-occurrence of S-virus (Bunyaviridae) in *M. depurator* experimentally infected with P-virus and Mari (1987) found co-occurrence of latent S_2-virus (Bunyaviridae) and occasionally 3-4 different viruses (S_2+W_2+τ, S_2+W_2+PC84, S_2+W_2+τ+PC84) in *Carcinus mediterraneus*, experimentally infected with W_2 (Reoviridae). Viral infections even occured in control animals which had received injections of sterile saline and were kept under sterile conditions (Mari, 1987). It therefore appears that latent viruses are present in wild populations, and outbreaks of disease can be triggered by stress (Mari, 1987). The occurrence of viruses in freshly captured crabs varies from 0.1 % in case of CHV of *C. maenas* in northern France (Bang, 1971) to 3 % for RhVA of *C. sapidus* around Chesapeake

Table 1.1.4 Viruses of crabs and crayfish

Virus	Family	Characteristics	Host species	References
Baculo-A virus	Baculoviridae?	Non-occluded, infects hepatopancreas, virion (incl. envelope) 60 x 270 nm, nucleocapsid (excl. envelope) 43 x 250 nm	*C. sapidus*	Johnson & Lightner (1988)
Baculo PP	Baculoviridae?	Ditto, virion 70 x 260 nm, nucleocapsid 40 x 200 nm,	*Paralithodes platypus* (king crab)	Johnson & Lightner (1988)
Scylla baculovirus (SBV)	Baculoviridae?	Ditto, virion 44x253 nm, nucleocapsid 24x205 nm	*Scylla serrata* (mud crab)	Anderson & Prior (1992)
Cherax baculovirus (CBV)	Baculoviridae?	Ditto, nucleocapsid 34x154 nm	*Cherax quadricarinatus* (freshwater crayfish)	Anderson & Prior (1992)
τ virus (Tau)	Baculoviridae	Ditto, nucleocapsid 63x310 nm	*Carcinus mediterraneus* (Mediterranean shore crab)	Pappalardo & Bonami (1979)
S virus	Bunyaviridae	Ovoid-filamentous, infects haemocytes, heart, midgut epithelium, virions 100 x 200 nm to 70 x 320 nm	*Macropipus depurator* (swimming crab)	Bonami & Vago (1971); Bonami (1980)
S_2 virus	Bunyaviridae?	Ditto, cytoplasmic in epithelial cells of all tissues and in haemocytes, virions 80 x 160 nm	*C. mediterraneus*	Mari & Bonami (1986)

CHV (crab haemocytopenic virus, Roscoff virus)	Bunyaviridae?	Spherical, cytoplasmic in haemocytes, 50-80nm	*C. maenas* (shore crab)	Bang (1971); Hoover & Bang (1978)
Herpes-like virus (HLV)	Herpesviridae?	Infects haemocyte nuclei	*Callinectes sapidus* (blue crab)	Johnson (1976)
Irido-like virus of *M. depurator* MdILV	Iridoviridae?	Diameter 170 nm, infects hepatopancreas	*M. depurator*	Montanie & Bonami (1993)
PC 84 virus	Parvoviridae	Naked, icosahedral, diameter 33 nm, nucleic in hepatopancreatic cells	*M. depurator*	Mari & Bonami (1986)
Chesapeake Bay virus (CBV)	Picornaviridae?	Diameter 30 nm, cytoplasmic in epithelial cells	*C. sapidus*	Johnson (1978)
P-virus	Reoviridae	Diameter 60 nm, paracryst. arrays in hepatopancreas, haemocytes, midgut epithelium	*M. depurator*	Montanie *et al.* (1993)
W virus	Reoviridae	Ditto,	*C. maenas*	Mari & Bonami (1986)
W_2 virus	Reoviridae	Ditto, commonly forms "rosettes" of 6 particles	*C. mediterraneus*	Montanie *et al.* (1993)
RLV-RhVA-disease: Reo-like virus (RLV)	Reoviridae?	Diameter 60 nm, paracrystalline arrays mainly in hyaline and semigranular haemocytes, co-occurs with RhVA	*C. sapidus*	Johnson (1977)
Rhabdo-like virus A (RhVA)	Rhabdoviridae?	30 x 200-600 nm, endoplasmic reticulum , co-occurs with RLV		

Bay and 4-20 % for Baculo A of *C. sapidus* from the gulf of Mexico (Johnson, 1983). The latter finding indicates that at least some viruses of crabs appear to be patchily distributed in the wild.

Most crustacean viruses have been described from penaeid shrimp, where stress or overcrowding appears to have led to outbreaks of latent viruses. With the intensification of shrimp aquaculture during the 1980s, viral diseases have become a major problem. Table 1.1.5 lists some important viral pathogens of penaeid shrimp. The first was discovered in the pink shrimp, *Penaeus duorarum* by Couch in 1974. This virus, named *Baculovirus penaei* (now known as PvSNP; Bonami *et al.*, 1995), has since been identified in many other species of penaeids (Table 1.1.5) and is one of the most devastating viral pathogens known in shrimp aquaculture (Fulks & Main, 1992). By contrast, another important virus, Monodon Baculovirus (PmSNP; Mari *et al.*, 1993) mainly infects the giant tiger prawn, *P. monodon* (Table 1.1.5). However, because *P. monodon* is the main species cultivated in Asia (Fulks & Main, 1992), outbreaks cause large economic losses and PmSNP probably contributed to the collapse of the Taiwanese shrimp industry, which declined by 80% in 1988 (Lin, 1989). The parvovirus IHHN is another virus which has caused significant mortalities in shrimp aquaculture (Fulks & Main, 1992) (Table 1.1.5). However, new viruses are constantly becoming prominent (Table 1.1.5). Thus, recent outbreaks of disease among white legged shrimp, *P. vannamei*, in Equador and Texas were caused by the, as yet unclassified, Taura syndrome virus (Hasson *et al.*, 1995), and in Japan, a newly discovered baculovirus has affected aquaculture of *P. japonicus* (Inouye *et al.*, 1996). Shrimp viruses appear to be endemic in adults which are captured as broodstock. Thus, Fegan *et al.* (1991) found PmSNV occlusion bodies in 5.7% of *P. monodon* broodstock in Thailand, whereas Couch (1976) found PvSNV in ca. 20% of adult *P. duorarum* in Florida. This implies that penaeid viruses can be distributed worldwide through the export and import of broodstock.

Table 1.1.5 Economically important viral pathogens of penaeid shrimp:

Virus	Family	Characteristics	Examples of affected species	Comment	References
Infectious hypodermal and hematopoietic necrosis virus (IHHN)	Parvoviridae	Occluded, diameter 20 nm	*P. japonicus, P. monodon* , *P. stylirostris, P. vannamei*	Present in aquaculture worldwide. Possibly endemic in wild *P. monodon* in SE Asia	Lightner *et al.* (1 Bonami *et al.* (19 Fulks & Main (1'
Hepatopancreatic parvo-like virus (HPV)	Parvoviridae	Ditto, diameter 22-24 nm	*P. esculentus, P. merguiensis, P. monodon, P. orientalis, P. semisulcatus*	Present in aquaculture worldwide	Lightner & Redn (1985); Bonami ((1995)
Lymphoidal parvo-like virus (LOPV)	Parvoviridae?	Diameter 25-30 nm	*P. monodon*	minor pathogen	Owens *et al.* (19
P. vannamei single nuclear polyhydrosis virus (former *Baculovirus penaei* (BP)-type virus) (PvSNP)	Baculoviridae	Occluded nucleocapsid 50 x 270 nm	*P. vannamei. P. aztecus, P. duorarum, P. marginatus, P. stylirostris, P. monodon*	Most likely several strains of this virus, endemic in wild Pacific and Atlantic populations	Couch (1974); Li *al.* (1992); Bonai (1995)
P. monodon SNPV (former Monodon baculovirus, MBV) (PmSNP)	Baculoviridae	Ditto, virion 75 x 300 nm	*P. monodon, P. merguiensis, P. plebejus*	Severely affects *P. monodon* worldwide, contributed to Taiwan shrimp aquaculture decline in 1988	Lightner & Redn (1981); Fulks & (1992); Mari *et a*

Baculoviral midgland necrosis (BMN)	Baculoviridae	Non-occluded, nucleocapsid ca. 75 x 300 nm	*P. japonicus*	Japan	Sano *et al.* (1981 & Main (1992)
Penaeid rod-shaped DNA virus (PRDV)	Baculoviridae	Non-occluded, nucleocapsid 84 x 226 nm	*P. japonicus*	Japan, causes penaeid acute viraemia (PAV)	Inouye *et al.* (19
Yellow head "baculovirus " (YHV)	unknown	RNA-virus	*P. monodon*	Severe losses in Thailand	Chantanachookir (1993); Wongtee *et al.* (1995)
Reo-like virus disease (REO)	Reoviridae	Diameter 50-70 nm	*P. japonicus, P. monodon*	Hawaii, Japan, Malaysia	Tsing & Bonami Anderson *et al.* (
Lymphoid organ vacuolization virus (LOVV)	Togaviridae	Virion ca. 36x160 nm	*P. monodon*	Minor pathogen	Spann *et al.* (199
Taura syndrome virus (TSV)	unknown	Non occluded, naked, icosahedra, diam. 31-32 nm	*P. vannamei*	Recent outbreaks in Colombia, Ecuador, Hawaii, Honduras, Peru	Hasson *et al.* (19 Lightner *et al.* (1

The exact mechanism of virus transmission to cultured shrimp is, as yet, unknown. Transoval transmission has been suspected but not been demonstrated conclusively. Diseases are absent or rare in the adults and mainly affect larval shrimp (Lightner, 1988; Overstreet *et al.*, 1988; Leblanc & Overstreet, 1990).

Several viruses of penaeids, such as PvNPV and IHHN, are common between a number of species (Table 1.1.5). Similarly, there are resemblances between viruses of different species of crabs, such as the S virus of *M. depurator* and S_2 of *C. mediterraneus* (Bunyaviridae), or W of *C. maenas*, P of *M. depurator*, W_2 of *C. mediterraneus* and the Reo-like virus of *C. sapidus* (all Reoviridae; Mari, 1987). Likewise, the baculoviruses, Baculo A of *C. sapidus*, τ of *C. mediterraneus* and *Scylla serrata* baculovirus share certain characteristics (Anderson & Prior, 1992) (Table 1.1.4). These similarities may indicate different strains of the same virus types (Anderson & Prior, 1992). Similarly, in penaids Lightner *et al.* (1992) proposed that PvSNP, PmSNP and HPV, which are endemic in populations from both the Pacific and Indian Oceans, represent distinct strains of their respective viral types. *In situ* hybridisation has shown that PvNPV isolates from Pacific and Atlantic penaeids differ (Lightner *et al.*, 1992). The information presented in Table 1.1.5 may therefore be misleading in that some shrimp viruses with broad host ranges in fact represent distinct host-specific viral strains.

The pattern of mainly opportunistic viral disease in crustaceans and their prevalence among the juvenile stages of penaeid shrimp resembles that described for diseases which are caused by free-living opportunistic pathogens (pages 6-11). However, the mechanism of viral disease is different because viruses appear to be carried as enzootics. Because outbreaks occur and opportunistic viral infections are frequently observed, enzootics do not result from viral benignness. Rather, the prevalence of viral outbreaks in the juvenile stages or in stressed animals appears to indicate that healthy normal crustaceans have some form of antiviral defence, which

can break down in adverse conditions. The part which either viruses or their host play in disease development and resistance may merit some further consideration, because viruses may be important for the regulation of wild populations. Not much is known about the impact of viruses on aquatic invertebrates, but studies on insects have indicated that viruses can play an important part in the regulation of their hosts (Tinsley & Harrap, 1978; Anderson, 1986; Zelasny & Alfiler, 1991). The nature of virus host interactions is therefore worthy of a brief consideration in the context of this thesis.

Virus-host relationships

The balance of virulence and resistance is a product of the dynamic interaction between a virus and its host (Anderson, 1986; Garnett & Antia, 1994). The host population density at which the virus can effectively persist depends on its rate of replication, which itself depends on both the duration of infectivity of the host and the probability of being transmitted to another susceptible host during that period (Anderson, 1986; Garnett & Antia, 1994). Essentially, virus-host interactions have to be regarded on two levels of within-host dynamics and between host dynamics (Garnett & Antia; 1994). On the level of within-host dynamics, a viral strategy will be to optimise infectiousness (Garnett & Antia; 1994). However, an endemic virus is likely to replicate slowly and not be very infectious (Anderson, 1986; Garnett & Antia, 1994). A more successful strategy appears to be an intermediate level of virulence which ensures reasonable infectivity without killing the host before transmission has occured (Anderson, 1986; Garnett & Antia, 1994). Thus, Scott *et al* (1994) have shown that moderate virulence by eastern equine encephalomyelitis virus against its vector, the mosquito, *Culisetes melanura*, increases transmission efficiency and viral fitness. Endemic viruses therefore do not automatically tend to become more benign (Anderson, 1986; Garnett & Antia, 1994; Scott *et al.*, 1994).

Virulence in turn is counteracted by the host, which is likely to evolve defence mechanisms or genetic resistance (Anderson, 1986). Increased resistance in host populations has been proposed in Californian oakworms, *Phryganidia californica,* after an outbreak of PcNPV (Martignoni, & Schmid, 1968) and observed in laboratory populations of the Indian meal moth, *Plodia interpunctella,* infected with a granulosis virus (Boots & Begon, 1993). The virus in turn mutates to circumvent host resistance. This results in co-evolution of host and virus, i.e. reciprocal genetic changes as result of ecological interaction (Futuyma & Slatkin, 1983). The emergence of genetically resistant strains of hosts or viruses, driven by host-virus coevolution, essentially divides the hosts and viruses into sub-populations. An example of host-virus coevolution in an invertebrate system is given by the sigma virus of the fruitfly, *Drosophila melanogaster*, which is endemic in 10-20 % of wild population of flies in Southern France (Fleuriet *et al*, 1990). Sigma virus is transmitted vertically, mainly transovally, but also through sperm (Tenninges, 1994). The virus is largely benign, but slightly reduces egg viability and overwintering fitness (Tenninges, 1994). Its benigness is mainly due to its inability to infect most tissues, including male sperm cells, after late in embryogenesis (Tenninges, 1994). In France, an increased prevalence of the type II strain, which is adapted to a resistance allele in the fruitfly population became apparent (Fleuriet *et al*, 1990). However, the transmission rate dropped, as the female flies in turn appeared to select males with viral clones which were less efficiently transmitted (Fleuriet *et al*, 1990). This therefore appears to be an example of on-going virus-host coevolution (Kilbourne, 1994).

Virus-host coevolution does not always lead to fragmentation of populations. Evolutionary theory predicts that, where parasites are transmitted through the germline, symbiotic relationships can develop. Because of the presence of several strains of viruses and host resistance alleles, such a symbiosis is not apparent in the *Drosophila*-σ-system. Among invertebrate viruses, the one known example are the

polydna viruses of ichmonid (Edson *et al.*, 1981) and braconid (DeBuron & Beckage, 1992) parasitic wasps. The parasitoids do not merely act as vectors, because the viruses do not replicate within host larvae (Stoltz, 1994). The viral genome is transmitted as part of the wasp genome and viral particles only form within the calyx of the oviduct of female wasps from where they are injected into host larvae during oviposition (Stoltz, 1994). In infected cells, products of the viral genome, rather than the formation of new virus particles, induce a reduction in the number of plasmatocytes, inhibition of monophenoloxidase and possibly other effects which result in inhibition of the encapsulation of the parasitoid egg (Webb & Luckhart, 1996). Upon hatching of the larva, virus gene transcription ceases and the host recovers part of its defence capabilities which may be required for its continued survival (Lavine & Beckage, 1996). The parasitoid larvae themselves are not attacked by host defences, possibly due to secretion of factors which prevent recognition (Lavine & Beckage, 1996). It might be speculated that the presence of the viral genome within the chromosome of the parasitoid and the absence of viral replication in host larvae or tissues other than the calyx would suggest that the "virus" is a component of the parasitoid itself, rather than a separate entity. Thus, "virus-like particles" which coat eggs of the parasitoid, *Venturia canescens* (Salt, 1973) or interfere with the immune response of *Drosophila* spp. larvae parasitized by the wasp, *Leptopilina heterotoma* (Rizki & Rizki, 1984) lack nucleic acids (Federsen *et al.*, 1986). Polydnaviruses, which contain DNA, are classified as viruses (Stoltz, 1994), but it is questionable that the symbiosis between polydnaviruses and parasitoid wasps is a result of host-virus coevolution.

In the Crustacea, vertical transmission of viruses has not been conclusively demonstrated, but S_2 virus of *C. mediterraneus* has been detected in the spermatophores and thus may be vertically transmitted (Mari, 1987). However, if this virus is also transmitted via the food chain, possibly through cannibalism, there

would be no basis for the evolution of symbiosis, although in this case viral latency is to be expected, at least until the host has reached adulthood.

At the level of between-host transmission, the rate of transmission within susceptible sub-populations is influenced by host density and distribution, which influences the frequency of encounters between infectious and suceptible individuals (Anderson, 1986; Garnett & Antia, 1994). Insects have provided particularly good examples as to how host distribution can influence viral replication strategies. In a patchy environment, a virulent virus can persist, because low host densities in foci of infection are replaced from outside (Anderson, 1986). This requires that the virus be transmitted either through time (by persisting in the environment until new hosts are recruited into the area) or through space (by wind, birds or parasitic vectors). Thus, many highly virulent nuclear polyhydrosis viruses form occlusion bodies which remain in the environment for years (Jaques, 1969). Similarly, occlusion bodies are formed by a range of crustacean viruses (Tables 1.1.4, 1.1.5), including PvNPV which is known to be patchily distributed in a population of adult *P. duorarum* in the Gulf of Mexico (Couch, 1974). However, this virus has little or no adverse effects on adult animals.

In patchy environments, viruses do not regulate the density of their hosts, but in an evenly distributed population, it is possible that viruses become key factors in density regulation. However, the relative importance of viruses can only be determined in a few cases, because of the importance of other environmental factors (Myers, 1988). The few outstanding examples are cases where viruses are introduced into populations in the absence of other natural enemies. The first observations of viral regulation of an insect pest were conducted on the spruce sawfly, *Gilpinia hercynae*, and its nuclear polyhydrosis virus, GhNPV (Balch & Bird, 1944). *G. hercyniae* spread into eastern Canada in 1930 and developed into a major pest in the absence of natural enemies, until the accidental introduction of GcNPV about 8 years

later (Balch & Bird, 1944). Within 4 years, the virus was regulating host numbers at minor levels (Balch & Bird, 1944). The virus is transmitted between feeding larvae through their faeces, and causes high mortalities (Balch & Bird, 1944). However, larvae which become infected shortly before pupation survive because the midgut cells, which are the only replication site of the virus, slough off and are replaced by undifferentiated cells of the pre-pupal gut which are resistant to infection (Balch & Bird, 1944). The virus remains in the lumen of the pre-pupal gut during diapause and recommences replication in the differentiated cells of the adult gut (Balch & Bird, 1944). The females transmit the virus on their eggs, which are laid single and up 1 mile apart, creating many small foci of infection (Balch & Bird, 1944). The virus persists in the evenly distributed population because it restricts virulence to certain life stages, which constitutes a form of viral latency. Furthermore, because it can persist in diapausing pupae for up to six years (Balch & Bird, 1944), the virus exploits the host's adaptation to yearly fluctuations in density. This further stabilizes the interaction, by smoothing temporal oscillations.

Viruses have shown promise in the control of forest caterpillars and can prevent outbreaks by pest species, although stable regulation is not achieved without re-application of the viruses (Myers, 1988). Similarly, the rhinoceros beetle, *Oryctes rhinoceros*, a pest of coconut palms throughout Asia and the Pacific islands, is controlled by a non-occluded baculovirus, described by Crawford (1994) as "the most useful insect pathogen known to man". This virus is more prevalent in the adults rather than in the larvae, but it is the adult beetles which cause the damage (Zelazny & Alfiler, 1991). The virus is transmitted through mating, and because the sexes attract each other, it can persist at an extraordinary low threshold density (Zelazny & Alfiler, 1991). Thus, release of the virus in Somoa and Tonga resulted in a reduction of pest density to as low as 5 beetles per ha (Crawford, 1994). Adults remain highly infective for about 3 weeks after ingestion of the virus and, because females do not begin oviposition until 2 months after emergence from the pupae,

infections of young adults severely reduce reproductive rates (Zelazny & Alfiler, 1991). However, the virus does not spread frequently to oviposition sites and is not passed through the pupal stage to adult beetles, so that healthy adults are continuously recruited into the population, allowing stable regulation by the virus (Zelazny & Alfiler, 1991).

From these examples in insects, it is apparent that viruses can play an important part in the ecology of their host species. Endemic viruses are present in adult crustaceans (e.g. Mari, 1987), and it could be speculated that similar regulation of crustaceans by viruses take place, but there is as yet no evidence of transmission pathways for viruses in the marine environment. There is some indication that certain viruses, such as the fish pathogen IPN, can be transmitted via the food chain and it is infectious for aquatic invertebrates (Halder & Ahne, 1988; Mortensen, 1993; Mortensen *et al.*, 1993). Transmission by crustacean scavengers, which are attracted to fish farms, may be responsible for outbreaks of IPN on the farms (Mortensen *et al.*, 1993). Molecular biology techniques could be used to determine the relatedness of viruses on different levels of the foodchain and in different species of crustaceans and perhaps resolve their transmission pathways, which will shed light on the ecology of host-virus interactions in the marine environment.

1.2 DEFENCE REACTIONS OF CRUSTACEANS

Introduction: Defence mechanisms and non-self recognition

Immunology as a discipline can be traced back to Metchnikoff's (1884) discovery of phagocytosis in a crustacean (*Daphnia*). Metchnikoff developed the concept that the immune response mediates the definition and maintenance of "self", defining the embryo during development and protecting the adult by destroying damaged cells (Tauber, 1994). By contrast, later work by Ehrlich and Koch emphasised the adaptive specific immunity against "non-self" (Tauber, 1994). Since then immunology has focussed mainly on the antibody-mediated specific immune response of vertebrates (Tauber, 1994). However, invertebrates lack antibodies and T-lymphocytes (Stewart, 1992) and in the context of this thesis, the word "immunity", when applied to invertebrates, is understood to refer to non-specific or semi-specific defence responses.

The absence of specific adaptive immunity in invertebrates has re-kindled the debate about the relative importance of "self" versus "non-self" in recognition. Burnet (1971) proposed that invertebrate immunity is mediated by "self" recognition and tolerance is mediated by the binding of immune cells to self-histocompatibility markers. This recognition of "self" is thought to underlie the non-fusion response of many colonial invertebrates (Burnet, 1971; Humphreys & Reinherz, 1994) and allograft rejection in echinoderms (Smith & Davidson, 1992, 1994). Alternatively, organisms may rely on the recognition of specific "non-self", such as bacterial lipopolysaccharides, peptidoglycans, fungal β-1,3 glucans, other microbial products or physical properties of foreign or damaged material (Söderhäll, 1992; Lackie, 1983; Ratcliffe *et al.*, 1984; Ratcliffe *et al.*, 1985). This semi-specific response against "natural antigens" is also found in the vertebrate "innate" immune response (Cohen, 1992).

Invertebrate immune defences comprise a range of non-specific or semi-specific mechanisms at different levels of the body. In metazoans, the primary protection is afforded by a physico-chemical barrier around the body (Ratcliffe *et al.*, 1985). Internally host defence is mediated by a number of fixed or circulating defence cells and extracellular factors (Ratcliffe *et al.*, 1985). Phagocytosis, has been regarded as the most primordial host defence, since it may be traced back to the protozoans. It is thought that the migratory amoebocytes in acoelomate invertebrates gave rise to circulating amoebocytes (Ratcliffe *et al.*, 1985). However, unicellular organisms can secrete defensive allochemicals, and, with the discovery of ciliate pheromones, which also act as autocrine growth factors, it has become apparent that cellular communication, mediated by humoral factors, may pre-date the advent of metazoans (Vallesi *et al.*, 1995). Furthermore, in animals with a circulatory system, many humoral immune factors may be derived from haemocytes (Ratcliffe *et al.*, 1985; Smith & Chisholm, 1992). Accordingly, cellular and humoral defence responses cannot be regarded as separate components within the invertebrate immune system (Smith & Davidson, 1992; Smith & Chisholm, 1992; Cooper *et al.*, 1994; Luporini *et al.*, 1994).

Physicochemical barriers to infection

The physico-chemical barrier around the body (mucus, cuticle, shell) and the gut-barrier (digestive juices, digestive enzymes and epithelial phagocytic cells) protect the body against invasion by pathogens (Ratcliffe *et al.*, 1985). Arthropods are protected by a tough chitinous cuticle, which also surrounds the foregut and hindgut. In crustaceans, a thin epicuticle of proteolipid material covers three chitineous layers. These comprise the calcified pigmented exocuticle, the calcified endocuticle and the non-calcified membraneous endocuticle (Stewart, 1993). The

Mechanical abrasion or enzymatic degradation of the epicuticle allows access by chitinoblastic microorganisms. These microbes are ubiquitous in the marine environment (Stewart, 1993). The epicuticle is constantly regenerated, but stress resulting from pollution or inadequate nutrition can impair epicuticle regeneration and trigger shell disease (Stewart, 1993). Some pathogens, such as the fungus, *Aphanomyces astaci*, are also able to break down the epicuticle and underlying layers (Stewart, 1993). Abrasions of the cuticle become associated with blackened areas which gave rise to names such as "black spot disease" or "Brandfleckenkrankheit" (Stewart, 1993). These are due to the deposition of melanin, a brownish to black pigment which is found in numerous invertebrate groups (Smith, 1996). The pigment affords camouflage, protection of the cuticle from UV radiation and may act in the sealing of injuries. It also has fungistatic properties (Söderhäll & Ajaxon, 1982). Furthermore, the formation of melanin generates reactive oxygen species (ROS) which are microbicidal (Riley, 1988), thus possibly acting in surface sterilisation of lesions (Smith, 1991).

Pathogens ingested with food may be destroyed by enzymes or other components of the digestive juice or be retained by the midgut epithelium of the host (Ratcliffe *et al.*, 1985). The concept of a "midgut-barrier" in insects arose from observations that much higher doses of viruses were required for oral infection than for infection via injection into the haemocoel (reviewed by Tinsley & Harrap, 1978). Similar observations have been made in crustaceans. Bonami (1980) found that swimming crabs, *Macropipus depurator*, orally infected with S-virus showed delayed mortality as compared to crabs which were infected via injection, and oral infection was unsuccessful in 20-40 % of individuals. Likewise, some specimens of *M. depurator* remained free of virus after oral infection with P-virus and mortality was 50-80 % as opposed to 100 % in crabs infected by injection (Bonami, 1980). In insects, components of the midgut barrier may include defensive cells of the gut lining (Ratcliffe *et al.*, 1985) and/or antimicrobial factors (Dickinson *et al.*, 1988;

lining (Ratcliffe *et al.*, 1985) and/or antimicrobial factors (Dickinson *et al.*, 1988; Funakoshi & Aizawa; 1989). Thus cecropin and lysozyme mRNA is induced in midgut cells of the tobacco hornworm, *Manducta sexta*, after injection with bacterial cell wall fragments (Dickinson *et al.*, 1988). Likewise, proteases in the gut juice of the silk worm, *Bombyx mori*, neutralize nuclear polyhydrosis virus *in vitro* (Uchida; 1984). It is not yet known whether or not the crustacean gut contains antimicrobial factors or defensive cells which protect the host from infection and neutralize viruses.

Crustacean haemocytes

Crustaceans contain three types of haemocytes, all of which act in host defence (Smith, 1991) (Table 1.2.1). It is unclear whether or not these are derived from the same progenitor cells which eventually mature to form granular cells (Smith, 1991). The hyaline cells are the primary circulating phagocytes (Smith & Ratcliffe, 1978; Smith & Söderhäll, 1983b; Söderhäll *et al.*, 1986). Semigranular cells, but not granular cells are also capable of phagocytosis. (Smith & Söderhäll, 1983a; Söderhäll *et al.*, 1986). The semigranular haemocytes discharge their granule contents *in vitro* in the presence of non-self components, such as bacteria (Smith & Söderhäll, 1983a), lipopolysaccharides (Söderhäll *et al.*, 1986) or β-1,3 glucans (Smith & Söderhäll, 1983b). The recognition of these microbial cell wall components by the semi-granular haemocytes affords a rapid ("acute") semi-specific response to infection. Degranulation releases components of the prophenoloxidase (proPO) cascade (see pages 37-40) and clotting factors. Components of the semi-granular cells also stimulate exocytosis by the granular haemocytes (Smith & Söderhäll, 1983a). Furthermore, lysates of granular haemocytes have been shown to contain antibacterial factors (Chisholm & Smith, 1992), which may be released

Table 1.2.1 Crustacean haemocytes

	Hyaline cells	Granular cells	Semigranular cells	References
size (*C. maenas*)	7 μm	8-10 μm	7 μm	Bauchau (1981)
granules	no	yes	yes (fewer than granular cells)	Bauchau (1981); Smith & Söderhäll (1983a); Söderhäll *et al.* (1986)
phagocytic	yes	no	yes	Bauchau (1981); Smith & Söderhäll (1983a); Söderhäll *et al.* (1986)
adhere to glass surfaces	yes	yes	yes	Smith & Söderhäll (1983a)
spreading	yes (long pseudopodia)	no	yes	Smith & Söderhäll (1983a)
contain prophenoloxidase (proPO) cascade?	no	yes	yes	Söderhäll & Smith, (1983).
contain opsonins	no	yes (linked to proPO cascade)	yes (linked to proPO cascade)	Söderhäll *et al.* (1994)
contain antibacterial factors	no	yes	probably	Chisholm & Smith (1992)

during degranulation. The cells themselves participate in the removal of injected bacteria by enclosing them in nodules which are deposited in the gills (Smith & Ratcliffe, 1981; White & Ratcliffe, 1982). Larger pathogens, such as fungi, are encapsulated (Unestam & Söderhäll, 1977). Nodule formation or encapsulation is often accompanied by melanization (Unestam, 1975; Smith & Ratcliffe, 1980a). This helps to physically seal off pathogens and may also contribute to microbial killing (White & Ratcliffe, 1982; White *et al.*, 1985). During degranulation, the granular and semigranular haemocytes further release opsonins which mediate phagocytosis by hyaline cells, indicating that the different haemocyte types cooperate *in vivo* (Söderhäll *et al.*, 1986).

The importance of haemocytes in antiviral defence of invertebrates is unclear. Earlier studies have shown that some species of molluscs (Acton & Evans, 1968; Cheng *et al.*, 1983), insects (Berheimer *et al.*, 1952) or crabs (Taylor *et al.*, 1964; McCumber & Clem, 1977) are capable of clearing viruses from the circulation, but it is unknown whether or not viral clearance is mediated through the haemocytes and whether viruses can bring about degranulation of granular or semigranular cells *in vitro*.

Fixed phagocytes

Ultimately pathogens and debris are removed from the haemolymph of crustaceans by fixed phagocytes, which are located in the heart, hepatopancreas and gills (Johnson, 1980; Smith & Ratcliffe, 1980a). In the shore crab, *C. maenas*, the nephrocytes of the gills act in the elimination of injected bacteria (Smith & Ratcliffe, 1980; White & Ratcliffe, 1982; White *et al.*, 1985) and in the mud crab, *Scylla serrata,* they may remove injected dyes (Mullaindhan, 1984) and probably also degrade non-self proteins which are subsequently removed to the hepatopancreas

(Mullainadhan & Ravindranath, 1984). In the blue crab, *Callinectes sapidus*, injected non-self proteins and some viruses accumulate in the gills, whereas other viruses accumulate in the hepatopancreas (McCumber & Clem, 1977). The fixed phagocytes may thus also participate in antiviral defence, but the fate of viruses in these tissues remains unclear. Activation of the fixed phagocytes in the hepatopancreas, indicated by increased vacuolization of the cytoplasm, can be triggered by bacteria or certain viruses (Johnson, 1980), but this organ is also the target organ for infection by many viruses of crustaceans (Johnson & Lightner, 1988). It therefore remains to be investigated whether or not the fixed phagocytes of crustaceans are able to sequester and inactivate viruses.

The respiratory burst

The ingestion of microorganisms by phagocytes is not necessarily accompanied by microbial killing (Mazet *et al.*, 1994). Indeed, phagocytosis may be of only limited value in the defence against intracellular parasites unless it is combined with additional defence reactions. Oxygen-dependent microbial killing by the respiratory burst involves the generation of reactive oxygen species (ROS), (Babior *et al.*, 1973; Badwey & Karnovsky, 1980). In mammals, the burst occurs if phagocytes ingest particles or bacteria (Badwey & Karnovsky, 1980) or encounter complement factors or various cytokines, often in response to viral infection (Maeda & Akaike, 1991). Membrane-associated NADPH oxidase of stimulated phagocytes produces superoxide anion (O_2^-) (Babior *et al.*, 1973), which is either converted to hydrogen peroxide (H_2O_2) spontaneously, is catalyzed by superoxygen dismutase, or is reduced by metal complexes to form the highly toxic hydroxyl radical ($\cdot OH$) (Fridovitch, 1978; Nappi *et al.*, 1995). The respiratory burst has been demonstrated in a range of invertebrates, including molluscs (Dikkeboom *et al.*, 1988; Pipe, 1992), crustaceans (Bell & Smith, 1993; 1994), echinoderms (Ito *et al.*, 1992) and tunicates

(Bell & Smith, 1994). However, the strength of the burst varies between phagocytic cells of different species (Bell & Smith, 1994) and the burst is not shown by phagocytes of all groups (Anderson *et al.*, 1973; Mazet *et al.*, 1994). Early studies by Anderson *et al.* (1973) reported that insect phagocytes were unable to exhibit a phagocytic burst. Likewise, Mazet *et al.* (1994) showed that phagocytes of the moth, *Spodoptera exigua,* do not generate ROS upon challenge with different elicitors and are unable to kill fungal cells or bacteria *in vitro*. In crustaceans, the respiratory burst has been demonstrated in hyaline cells of *C. maenas* (Bell & Smith, 1993; 1994), but the response is weaker than that recorded for fish or mammals. In mammals, several viral diseases result in the generation of ROS by phagocytic cells (Maeda & Akaike, 1991). During viral infection, phagocytes appear to be activated by interferons, tumor necrosis factor, virus specific antibody or complement factors (Maeda & Akaike, 1991). Human immunodeficiency virus (Kimura *et al.*, 1993) or influenza-A virus (Kazhdan *et al.*, 1994) also directly elicit a respiratory burst in human phagocytes *in vitro*. In the case of influenza-A, the response remains contained within the neutrophil and release of O_2^- is not detected (Kazhdan *et al.*, 1994). It is unknown whether or not viral infection in invertebrates similarly triggers ROS formation.

The ROS generated during viral infection can be dangerous to the host (Maeda & Akaika; 1991). They are the principal factors causing mortality in mice infected with influenza virus, where mortality occured 5-6 days after the virus was cleared and could be considerably reduced by the injection of conjugated antioxidant enzymes (Malda & Akaika, 1991). In *C. maenas* the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase are present within the haemocytes and, to some extent, the plasma (Bell & Smith, 1995), but it is not known whether or not the levels of antioxidant enzymes levels increase during viral infection.

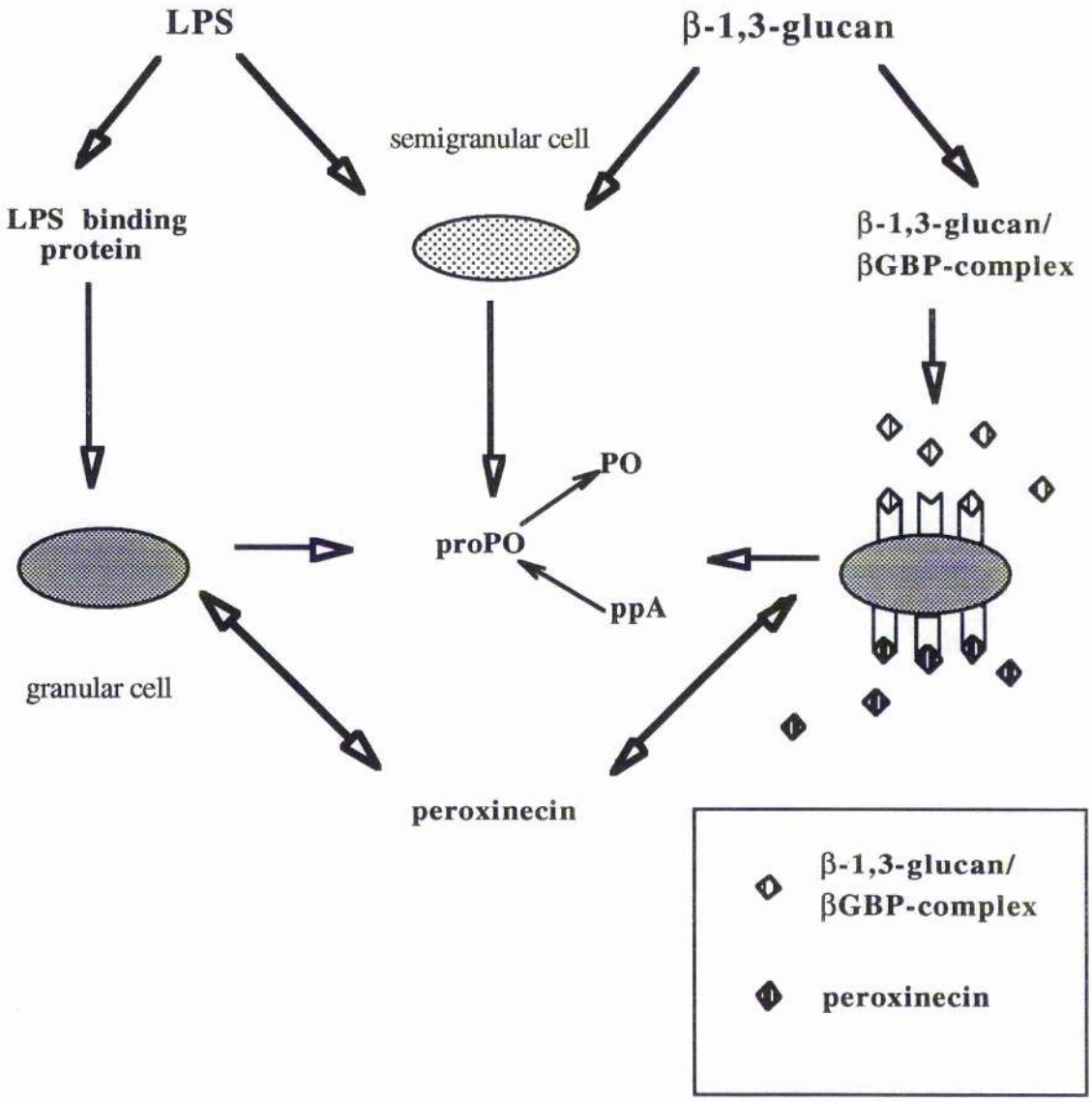
The proPO cascade

The prophenoloxidase (pro-PO) cascade is a cascade of serine proteases, which is activated by exposure to fungal β-1,3 glucans (Unestam & Söderhäll, 1977), lipopolysaccharides (LPS) of Gram negative bacteria (Smith & Söderhäll, 1983; Söderhäll & Häll, 1984), or bacterial peptidoglycans (Smith & Söderhäll, 1983; Söderhäll *et al.*, 1986). In crustaceans, components of the proPO cascade mediate a range of defence reactions *in vitro* (reviewed by Johanssen & Söderhäll, 1989; Smith, 1991; Smith, 1996). These include phagocytosis (Smith & Söderhäll, 1983; Söderhäll *et al.*, 1986; Thörnqvist *et al.*, 1994), cell adhesion (Johansson & Söderhäll, 1988), encapsulation (Smith *et al.*, 1984; Söderhäll *et al.*, 1984; Kobayashi *et al.*, 1990) and clotting (Söderhäll, 1981).

The proPO cascade has been studied in detail in the crayfish, *Pacifastacus leniusculus*, and is illustrated in Figure 2.1. Peptidoglycans, LPS or β-1,3 glucans trigger degranulation of the granular and semigranular haemocytes and the release of several serine proteases together with proPO and a 76 kDa glycoprotein (Söderhäll, 1992) (Figure 1.2.1). The 76 kDa glycoprotein, peroxinecin, is a peroxidase (Johansson *et al.*, 1995) which has multiple functions in the crayfish immune response (Söderhäll, 1992; Söderhäll *et al.* 1994). It causes adhesion of granular and semigranular haemocytes (Johansson & Söderhäll, 1988), promotes encapsulation of parasites (Kobayashi *et al.*, 1990) and acts as an opsonin for phagocytosis by the hyaline cells (Thörnqvist *et al.*, 1994). It also binds to the granular cells and thus generates a feedback-loop of cell degranulation (Johansson *et al.*, 1995) (Figure 1.2.1). It rapidly degrades once it has been excreted into the haemolymph (Johansson & Söderhäll, 1989), ensuring that the maximum response remains concentrated around the site of infection. The proPO response is further modulated by serine

Figure 1.2.1 Activation of the proPO cascade in the crayfish, *P. lenisculus* (original drawing). The cascade is activated by LPS, peptidoglycan (not shown) or β-1,3 glucans, which cause degranulation of the granular and semigranular cells (Söderhäll, 1992). Degranulation of the former involves binding proteins. Activation of the granular cells by β-1,3 glucans is illustrated on the right of the figure. The 100 kDa β-1,3 glucans binding protein (βGBP) (Duvic & Söderhäll, 1990), a plasma glycolipoprotein (Cerenius *et al.*, 1994; Hall *et al.*, 1995), binds to a 340 kDa receptor on the granular haemocytes (Duvic & Söderhäll, 1992), once it has formed a complex with β-1,3 glucans. The receptor contains a putative recognition site for an RGD (Arg, Gly, Asp), which may indicate that βGBP is similar to the integrin family in vertebrates (Söderhäll *et al.*, 1994). Degranulation leads to the release of several serine proteases together with proPO and a 76 kDa glycoprotein (Söderhäll, 1992). The proPO of *P. lenisculus* is an 80 kDa protein (Aspán *et al.*, 1995), which is cleaved by ProPO activating enzyme (ppA), a 36 kDa serine protease, to yield a 62 kDa phenoloxidase (Aspán *et al.*, 1990). The 76 kDa glycoprotein, peroxinecin (Johansson *et al.*, 1995), causes adhesion of granular and semigranular haemocytes (Johansson & Söderhäll, 1988), promotes encapsulation (Kobayashi *et al.*, 1990) and acts as an opsonin (Thörnqvist *et al.*, 1995). It also binds to the 340 kDa granulocyte receptor via a KGD (Lys, Gly, Asp) sequence, thus generating a feedback-loop of cell degranulation (Johansson *et al.*, 1995).

Figure 1.2.1


LPS
β-1,3-glucan
semigranular cell
LPS binding protein
β-1,3-glucan/ βGBP-complex
PO
proPO
ppA
granular cell
peroxinecin
β-1,3-glucan/ βGBP-complex
peroxinecin

protease inhibitors such as a 155 kDa ppA inhibitor (Hergenhahn *et al.*, 1987), an α-macroglobulin with similarity to vertebrate macroglobulins (Hergenhahn *et al.*, 1988) and the 45 kDa crayfish serpin PAPI 2 (Liang & Söderhäll, 1995).

The importance of the proPO cascade in antiviral defence is largely unknown. In insects, Miranpuri *et al* (1992) have observed that PO activity peaks at day 4 after oral infection of the migratory grasshopper, *Melanoplus sanguipipes*, with an entomopoxvirus. This event appears to coincide with the virus crossing the gut barrier (Miranpuri *et al.*, 1992). Furthermore, Ourth & Renis (1993) have found that melanization by PO from the haemolymph of the tobacco budworm, *Heliothis virescens*, causes neutralisation of Herpes simplex virus 1 and vesicular stomatitis virus. However, it has not been investigated whether or not viruses can bring about activation of the proPO cascade *in vitro*.

Clotting

Injuries to the cuticle or integument need to be sealed quickly to avoid entry of pathogens and, in coelomate animals, loss of blood or body fluids. All animals have a wound healing response which involves the aggregation of cells at the site of injury (Ratcliffe *et al.*, 1985). In coelomate animals, rapid clotting is often accompanied by collagen formation (Ratcliffe *et al.*, 1985). In molluscs and annelids, this response is combined with muscular contraction to seal the wound, but in animals with hard tests or exoskeletons, rapid wound sealing must be achieved by other means (Ratcliffe *et al.*, 1985). Uniquely, in arthropods (with the exception of dipterans) clotting also involves plasma gelation (Ratcliffe *et al.*, 1985).

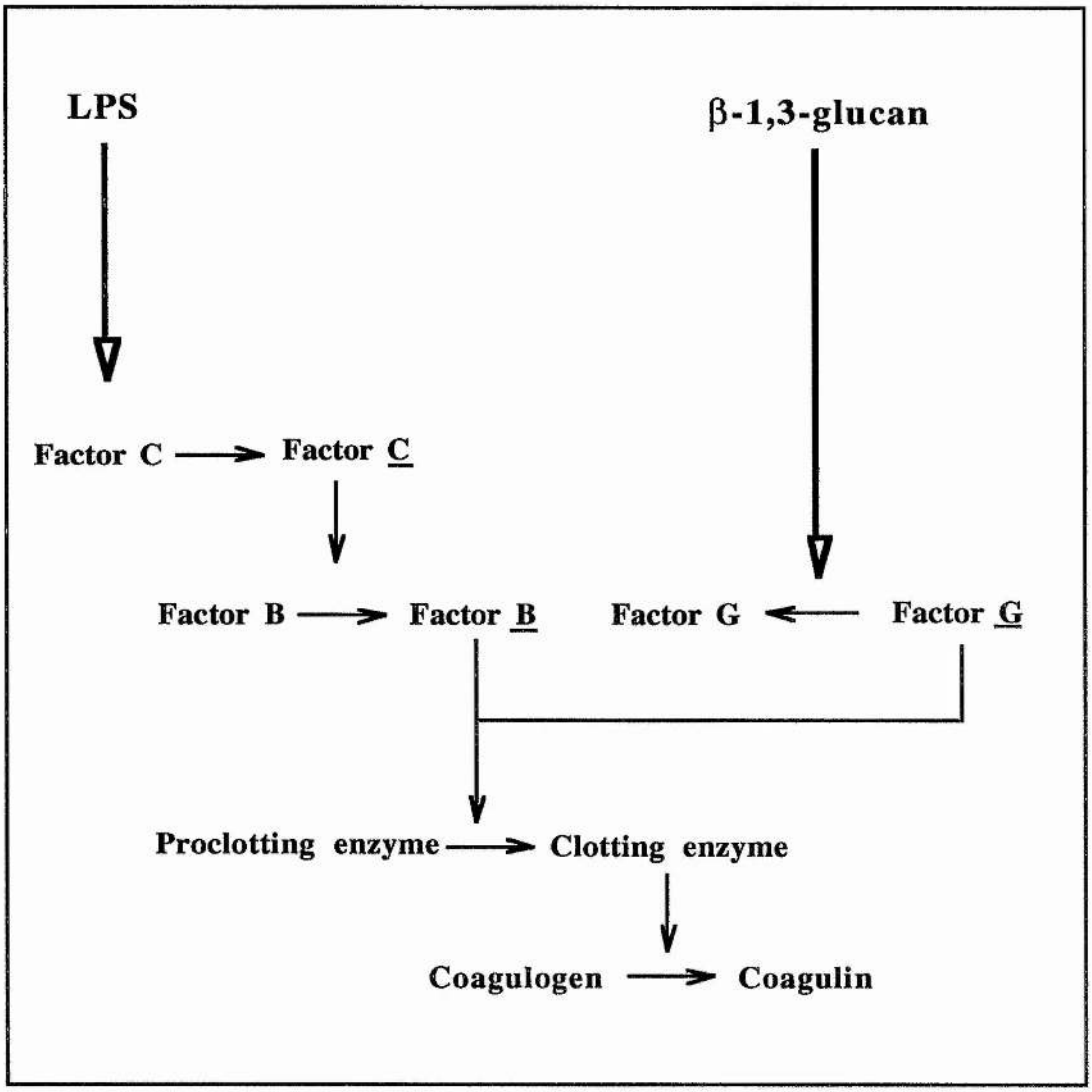
The clotting pathway of *Limulus polyphemus* has been studied in detail (reviewed by Iwanaga, 1993 and Iwanaga *et al.*, 1994). It involves serine protease

cascades which bear certain similarities to the proPO-mediated response in insects and crustaceans. However, horseshore crabs lack phenoloxidase as final component of the cascade (Smith & Söderhäll, 1986b). Structural homologies of *Limulus* factors to components of the proPO cascade have not yet been shown, so that it is unclear whether or not the two systems are evolutionary related. However, there are similarities between *Limulus* factors and mammalian serine proteases and transglutaminases (Iwanaga, 1993 and Iwanaga *et al.*, 1994).

Figure 1.2.2 illustrates the *Limulus* clotting reaction, as described by Iwanaga (1993) and Iwanaga *et al.*, (1994). The clotting factors are situated in the large granules or cytosol of the granulocytes, the main haemocyte type in horseshoe crabs and clotting is triggered upon exocytosis in response to infection (Iwanaga; 1993; 1994). As with the proPO cascade of insects and crustaceans, the *Limulus* clotting response is controlled by two separate serine protease pathways, one triggered by LPS, the other by β-1,3 glucans (Iwanaga; 1993; 1994) (Figure 1.2.2). Both pathways culminate in the conversion of a proclotting enzyme into a clotting enzyme (Iwanaga; 1993; 1994) (Figure 1.2.2). The clotting enzyme converts coagulogen, a cationic 19.7 kDa polypeptide, functionally, but not structurally, similar to vertebrate fibrinogen, into coagulin (Iwanaga; 1993; 1994). The clotting enzyme cleaves a 28 amino acid peptide, peptide C, from the central part of this molecule, leaving two chains, A (18 residues) and B (129 residues), linked by two disulphide bridges (Iwanaga; 1993; 1994). These chains polymerize into a coagulin gel, catalyzed by a 87 kDa Ca^{2+}-dependent transglutaminase (TGase) (Iwanaga; 1993; 1994). In this manner, the *Limulus* TGase acts in the same manner as the mammalian plasma factor XIIIa, which catalyses the cross-linking of fibrin to itself and other proteins (Iwanaga; 1993; 1994). It also has 34.7 % similarity to human factor XIIIa subunit, but has a cationic amino-terminal extension of 60 residues unknown from

Figure 1.2.2 Activation of the *Limulus* clotting pathway (redrawn from Iwanaga; 1993; 1994). The LPS-mediated pathway (left) has two intermediate zymogens, Factor C (converted to factor C) and Factor B (converted to factor B) (Iwanaga; 1993; 1994). Factor C is a 123 kDa glycoprotein, composed of a 43 kDa light chain and a 80 kDa heavy chain (Iwanaga; 1993; 1994). In the presence of LPS or lipid A, the light chain is cleaved into chains of 8.5 kDa and 34 kDa (Iwanaga; 1993; 1994). the latter is a serine protease with 36.7% identity to human α-thrombin (Iwanaga; 1993; 1994). The second zymogen of the LPS pathway, factor B, is a single-chain 64 kDa glycoprotein which is converted to factor B, comprising a 32 kDa chain and a 25 kDa chain (Iwanaga; 1993; 1994). By contrast, the β-1,3 glucan-mediated pathway (right) has only one intermediate zymogen, factor G, which consists of two subunits of 72 kDa (a) and 34 kDa (b) (Iwanaga; 1993; 1994). Subunit a) is apparently a glucanase, whereas subunit b) is a serine protease zymogen with 40.5% identity to factor B (Iwanaga; 1993; 1994). Both factor G and factor B convert the proclotting enzyme, a 54 kDa glycoprotein, into an active enzyme (Iwanaga; 1993; 1994).

Figure 1.2.2



(redrawn from Iwanaga; 1993; 1994)

mammalian TGases (Iwanaga; 1993; 1994). The clotting response is regulated by *Limulus* serpin, a 48 kDa glycoprotein, which has 39 % identity to human monocyte/neutrophil elastase inhibitor (Miura *et al.*, 1994). It inhibits clotting enzyme and removes it from the plasma by binding to specific haemocyte receptors (Miura *et al.*, 1994). In addition, a 185 kDa α- macroglobulin is also secreted by the cells (Armstrong *et al.*, 1990).

In crustaceans, coagulating factors are present in both haemocytes and plasma. However, with the exception of the parasitic rhizopod, *Sacculina carcini* (Levin, 1967), plasma coagulation also requires factors derived from granular or semigranular haemocytes (Smith & Söderhäll, 1986b). Gelation of haemocyte lysate supernatants can be achieved by triggering cellular serine proteases with β-1,3 glucans, LPS or peptidoglycans, demonstrating that the clotting response is linked to the proPO cascade (Söderhäll, 1981; Söderhäll & Smith, 1986b; Smith, 1991; Smith & Chisholm, 1992). An important difference between the clotting pathways in horseshoe crabs and crustaceans is that, in the latter, proteolytic conversion of coagulogen is not required (Fuller & Doolittle, 1971; Martin *et al.*, 1991). Although functionally similar to *Limulus* coagulogen, the crayfish clotting protein is different in consisting of a 2 subunits of 210 kDa (Kopaçek *et sl.*, 1993). The modulation of the clotting response by *Limulus* serpin and α- macroglobulin bears some resemblance to the inhibition of proPO activation in the crayfish (page 40).

Apart from wound sealing, the clotting response may have direct functions in the defence against microorganisms. Thus, in *Limulus*, the TGase also incooperates other plasma proteins and helps to immobilise microorganisms (Iwanaga; 1993; 1994). Furthermore, by creating a viscous environment around degranulating haemocytes, clotting helps to increase the local concentration of antimicrobial factors released during degranulation (Iwanaga; 1993; 1994). However, it is not known whether antimicrobial factors in *Limulus* haemocytes (reviewed by Iwanaga;

1993; 1994), or crustacean granular cells (Smith & Chisholm, 1993; 1994) act in antiviral defence *in vivo*.

Cytotoxicity

Cytotoxicity is the cell-mediated lysis of foreign cells by host effector cells. This response is present in several species of invertebrates (reviewed by Ratcliffe *et al.*, 1985; Roch, 1996). Possible functions of cytotoxicity in invertebrates include the maintenance of integrity in colonial invertebrates and the destruction of parasites in molluscs and probably other groups (Ratcliffe *et al.*, 1985). In mammals, natural killer (NK) cells are of considerable importance in the removal of transformed cells and virus-infected cells (Janeway, 1989). A patient who lacks NK cells, may be killed within days after primary infection with herpes simplex virus, usually regarded as only mildly pathogenic, because proliferation of antibody-mediated killer T-cells does not occur until late in infection (Janeway, 1989). It is possible that cytotoxic cells likewise mediate lysis of virus infected cells in invertebrates but this is, as yet, unknown.

The mechanism of cytotoxicity has been studied in sipunculids (Boiledieu & Valembois, 1977), molluscs (Yoshitho, 1988) and, in greater detail, in the tunicate *C. intestinalis* (Peddie & Smith, 1994a, 1994b). In all these species, cytotoxicity requires direct contact between target and effector cell (Boiledieu & Valembois, 1977; Yoshino, 1988; Peddie & Smith, 1994b). In the tunicate, *C. intestinalis*, target-effector cell conjugation is Ca^{2+} and Mg^{2+} dependent, whereas lysis is Ca^{2+} dependent (Peddie & Smith, 1994b). Cell lysis is inhibited by sodium azide, cytochalasin B, colchicine, vinblastine and monesin, showing that the phenomenon requires active metabolism, an intact cytoskeleton and that lysis is mediated by secretion (Peddie & Smith, 1994b). By contrast, the reaction is not inhibited by

superoxide dismutase or catalase, indicating that O_2^- or H_2O_2 are not involved (Peddie & Smith, 1994b).

Intact microtubules and the presence of calcium ions are also required in cytotoxicity mediated by sipunculid cells (Boiledieu & Valembois, 1977) and, in sipunculids (Boledieu & Valembois, 1977) and molluscs (Yoshino, 1988), lysis is probably mediated by phospholipases. The cytotoxic response in these species is therefore similar to that of natural killer cells (NK) in mammals. Moreover, cytotoxicity in *C. intestinalis* seems to be mediated through lymphocyte-like cells, which are structurally similar to vertebrate lymphocytes (Peddie & Smith, 1995). However, such "lymphocyte-like" cells have not been reported from other invertebrate groups. In the crayfish, *Astacus astacus*, cytotoxicity is mediated through granular and semigranular cells and contact with host cells is required for this response (Söderhäll *et al.*, 1985). However, as haemocyte lysate supernatants from the shore crab, *Carcinus maenas*, contain haemolytic activity against mouse erythrocytes *in vitro* (Smith & Söderhäll, 1983b), it cannot be ruled out that lysis of the target cells resulted from factors released during cell degranulation. It is not known whether or not cytotoxicity in crustaceans is directed at virally infected cells.

Agglutinins

Agglutinins are factors which bring about the aggregation of foreign particles such as bacteria, parasites or red blood cells (reviews by Ratcliffe *et al.*, 1985; Smith & Chisholm, 1992). These factors are found in the haemolymph of many invertebrates and also in plants and the serum of vertebrates. In mammals, they may augment the action of immunoglobulins (Ratcliffe *et al.*, 1985). Most of the agglutinins which have been characterised so far are proteins with recognition sites

Table 1.2.2 Invertebrate lectins (based on Vasta *et al.*, 1994)

	C-type lectins	S-type lectins	Pentraxins	others
Ca^{2+} dependence	yes	no	yes	yes
Carbohydrate specificity	Mostly β-galactosides	variable	phosphocholine, sialic acid, galactans	variable
Structure	variable, but similar carbohydrate recognition domains (120-130 residues) (Drickamer, 1988)	Most invertebrate S-type lectins characterized so far are galaptins which have 14 kDa subunits (Vasta *et al.*, 1994)	1.0×10^6 Da, 6 subunits in *Limulus* (Liu *et al.*, 1994)	variable
Cysteines	disulphides	require free thiols for binding	disulphides	disulphides
Examples	Lectins of the acorn barnacle, *Megabalanus rosa* (Muramoto & Kamia, 1990)	Tunicate, *Clavelina picta* (Vasta *et al.*, 1994) and sponge, *Geodia cydonum* (Pfeiffer *et al.*, 1993). Not yet described from crustaceans.	Limulin from the horseshoe crab (*Limulus polyphemus*) (Liu *et al.*, 1994), lectins from the tunicates *Didemnum candidum* (Vasta *et al.*, 1986 and *Clavelina picta* (Elola & Vasta, 1994). Not yet described from crustaceans.	Lectins from the sponge, *Axinella polypoides* (Buck *et al.*, 1992). Not yet described from crustaceans.

(usually 2 or more) for specific carbohydrates (Ratcliffe *et al.*, 1985; Smith & Chisholm, 1992). These molecules, excluding carbohydrate-specific immunoglobulins, are collectively known as lectins (Sharon & Lis, 1972; Barondes, 1984). Invertebrate lectins have been classified into three major groups, which are listed in Table 1.2.2. However, the structure of many invertebrate lectins is, as yet, unknown (Vasta *et al.*, 1994). In the Crustacea, most of the lectins that have been characterized so far are calcium dependent (Chisholm & Smith, 1992; Vargas-Albores *et al.*, 1993; Nalini *et al.*, 1994). However, sequence data are only available for lectins of the acorn barnacle, *M. rosa* (Muramoto & Kamia, 1990).

It is thought that agglutinins fulfill a range of functions (Ratcliffe *et al.*, 1985), but the importance of agglutinins in host defence of crustaceans is unclear (Smith & Chisholm, 1992). Many crustacean agglutinins act on bacteria or microorganisms, which may be important for defence in some species, but in others, resistance against microorganisms is not linked to agglutinating activity (Smith & Chisholm, 1992). In the blue crab, *Callinectes sapidus,* neutralising activity against the coliphage T_2 is distinct from haemagglutinating activity (Mc Cumber *et al.*, 1977). Therefore, agglutinins do not appear to be involved in the direct inactivation of viruses. Renwrantz (1986) questioned the efficiency of agglutination in host defence because titres of pathogens *in vivo* are likely to be low. He, and other workers, proposed that agglutinins act primarily as opsonins (reviews by Ratcliffe *et al.*, 1985; Renwrantz, 1986; Chisholm & Smith, 1992). However, purified haemagglutinins of the lobster, *Homarus americanus*, did not increase the rate of phagocytosis by the haemocytes *in vitro* (Hall & Rowlands, 1974). Many infected cells display viral glycoproteins on their surfaces and in vertebrates, these are the prime targets for opsonisation of infected cells, although this response tends to be antibody-mediated (Nash, 1996). An opsonisation of virally infected cells in invertebrates could be achieved by lectins which recognize viral carbohydrates, but targeting of virally infected cells in invertebrates has not been investigated.

In mammals, a mannose binding C-type lectin in guinea pig serum mediates the complement-induced lysis of guinea pig cells infected with influenza virus by binding to a high mannose oligosaccharide at the tip of the viral haemaglutinin (Reading *et al.*, 1995). Mammalian mannose binding protein (MBP) has separate binding sites for C1r and $C1s_2$ and activates the classic complement pathway, probably by mimicking C1q (Schweinle *et al.*, 1990). Likewise, in invertebrates, some lectins act in the activation of defence pathways, such as the *Limulus* clotting reaction or insect proPO cascade (Muta *et al.*, 1991; Chen *et al.*, 1995). Thus, the *Limulus* factor C, which initiates the LPS-activated clotting pathway, has separate lectin-like and serine protease-like domains (Muta *et al.*, 1991).

In insects, Chen *et al.* (1995) have shown that the lectins BDL 1, BDL 2 and GSL from the cockroach *Blaberus discoidalis* activate its proPO cascade to the same extent as laminarin and enhance laminarin activation of the cascade. The lectins appear to bind to mannose (BDL 1), N-acetylglucoseamine (BDL 2) or galactose residues on different haemocyte receptors (Chen *et al.*, 1995). These results are interesting because the haemocytes of the migratory grasshopper, *Melanoplus sanguinipes,* show increased binding of wheat germ agglutinin (WGA) and concanavalin A (conA) after infection with an entomopoxvirus (MsEPV) (Miranpuri *et al.*, 1993). This response may be indicative of an increase in cell membrane carbohydrates, although binding of WGA could be due to the presence of MsEPV occlusion bodies, which bind strongly to this lectin (Miranpuri *et al.*, 1993). However, an increase in membrane carbohydrates was also reported by Nappi & Silvers (1984) for a temperature-dependent mutant of *Drosophila melanogaster* (*Tum'*) which spontaneously forms melanotic tumours against endogenous tissues. Nappi & Silvers (1984) found that immune reactivity of haemocytes, which in *Tum'* is present in flies kept at 29°C but not 21°C, was linked to increased labelling of lamellocytes with WGA. Similarly, in crustaceans, Kobayashi & Söderhäll (1990) reported increased binding of ConA to the surface of granular haemocytes in the freshwater crayfishes *Astacus astacus,* infected with *Psorospermium haeckelii*, and

Pacifastacus lenisculus, infected with *Aphanomyces astaci.* It remains to be seen whether or not viral infections in crustaceans also trigger changes in haemocyte membrane carbohydrates and whether crustacean lectins activate the proPO cascade. This could indicate a defence response which is directed against infected cells, rather than the viruses themselves.

Killing factors

Pathogens which have been recognized by host defence responses must be removed or rendered inactive. Invertebrates have a number of molecules which kill pathogens (reviews by Smith, 1991; Chisholm & Smith, 1992; Boman, 1995; Roch, 1996). These can be grouped into lytic factors, antimicrobial peptides, antimicrobial proteins and reactive oxygen species, which are generated by processes such as the phagocytic burst (Babior *et al.*, 1973) (see pages 35-36) or melanization (Riley *et al.*, 1988).

a) Lytic factors

Lysozyme cleaves the β-1,4 glycosidic links in cell walls of Gram positive bacteria. In insects, it is induced upon bacterial infection along with a range of other immune factors (Russell & Dunn, 1996) and is thought to act synergistically with some insect antibacterial peptides and proteins, e.g. as cecropins and attacins (Engström *et al.*, 1984; Chalk *et al.*, 1994). Lysozyme has been detected in some crustaceans (Fenouil & Roch, 1991), although not in the shore crab, *C. maenas* (Smith & Chisholm, 1992). Lysozyme has no known effect on viruses.

Cytolytic molecules are present in a range of invertebrates (Roch, 1996). Haemolysins are heat labile, Ca^{2+} dependent proteins, consisting of 40-60 kDa subunits, which form pores in the membranes of erythrocytes and may bind to bacteria and zymosan (Roch, 1996). In annelids, haemolysins are also antibacterial

and act as agglutinins (Roch *et al.*, 1989), whereas in echinoderms, haemolysins appear to act in the cytotoxic response (Canicatti, 1991). In addition to haemolysins, some small peptides, such as bee melittin and metriolysin from the anemone *Metridium senile* also act cytolytically (Bernheimer & Rudy, 1986). Furthermore, earthworms (*Lumbricus terrestris*), contain a non-proteinaceous haemolytic agent which belongs to the saponins, thermostable glycosides or phosphorylglyceryl agents which are also found in leeches and biting insects (Roch, 1996). In crustaceans, weak lytic activity against mouse RBC has been detected in HLS and serum of *C. maenas* (Smith & Söderhäll, 1983b), but lytic activity is not linked with antibacterial activity (Chisholm, 1994). The function of lytic factors in crustaceans remains unclear. They are unlikely to be of importance in antiviral defence.

b) Antimicrobial peptides

Antimicrobial proteins of less than 10 kDa are regarded as antimicrobial peptides (Boman, 1995). Such small molecules are of particular value in antimicrobial defence, because they are rapidly synthesized, without involving specialized cells or tissues, and have rapid diffusion rates (Boman, 1991). Antimicrobial peptides are also mostly not toxic to eukaryotic cells and do not cause lysis of bacteria which could lead to sepsis (Boman, 1991). Furthermore, many are active against a wide range of bacteria, as well as other microorganisms, including viruses (Boman, 1995). In the past decade, antimicrobial peptides have attracted much attention because of their perceived importance for host defence and their potential use as chemotherapeutic agents (Boman 1995). Over different 70 antimicrobial peptides had been identified by 1995 and new forms are continuously being discovered (Boman, 1995). Most attention has focussed on insects and mammals (reviews by Cociancich *et al.*, 1994; Boman, 1995; Hoffmann, 1995; Selsted & Ouellette, 1995; Zanetti *et al.*, 1995), but these molecules are widespread in the animal kingdom and have also been detected in horseshoe crabs (Muta *et al.*,

1987; Miyata *et l.*, 1989; Saito *et al.*, 1995), scorpions (Cociancich *et al.*, 1993), birds (Harwig *et al.*, 1994), skin secretions of frogs (review by Barra & Simmaco, 1995) and body fluid of a nematode, *Ascaris suum* (Kato, 1995). There are important homologies between sequences or secondary structures among some peptides from different animal groups, indicating that they have been conserved in evolution (Boman, 1995). In spite of the apparent importance of these molecules in host defence (Cociancich*et al.*, 1994; Boman 1995; Hoffmann, 1995; Zannetti *et al.*, 1995), antimicrobial peptides have not, as yet, been characterized from the Crustacea.

Antimicrobial peptides fall into five major chemical and structural categories (Boman, 1995), which are given in Table 1.2.3, along with examples from different animal groups. Insects, in particular, produce a wide range of antimicrobial peptides in response to bacterial challenge (Cociancich *et al.*, 1994; Boman, 1995; Hoffmann, 1995). The first to be described were cecropins, which were isolated from the silk moth, *Hyalophora cecropia*, (Hultmark *et al.*, 1980; Steiner *et al.*, 1981) and are present in many species of Lepidoptera and Diptera (Cociancich *et al.*, 1994a). Cecropins have also been isolated from pig intestine (Lee *et al.*, 1989). Similarly, insect defensins are likewise widely distributed, although they appear to be lacking in the Lepidoptera (Cociancich *et al.*, 1994a). Defensins and related molecules have been identified in a range of mammals and in chickens (review by Selsted & Ouellette, 1995). In insects, antibacterial peptides are induced by bacterial challenge (Cociancich *et al.*, 1994; Boman, 1995; Hoffmann, 1995), whereas in mammals they are constitutive, mostly within polymorphonuclear neutrophils (Boman, 1995; Zannetti *et al.*, 1995). Similarly, haemocyte granules of horseshoe crabs (*Limulus* spp., *Tachypleus* spp.) contain the antibacterial peptides tachyplesins, polyphemusins (Miyata *et l.*, 1989) and big defensin (Saito *et l.*, 1995). These are released during the clotting response (Iwanaga *et al.*, 1994). In crustaceans, increased antibacterial activity after challenge with bacteria has been detected in the plasma and serum of

spiny lobsters, *Panulirus* spp. (Evans et al., 1968; 1969) and the American lobster, *Homarus americanus* (Acton et al., 1969; Stewart & Zwicker, 1972; Mori & Stewart, 1978), but it is unclear whether or not this response is due to the *de novo* synthesis of killing factors, including antimicrobial peptides, or due to release of constitutive factors from the haemocytes (Stewart & Zwicker, 1972). In *C. maenas*, and several other species, antibacterial activity has been detected in haemocyte lysate supernatants (HLS) without prior challenge with bacteria (Chisholm & Smith, 1992; 1994). At least one of the active components in *C. maenas* HLS appears to be a peptide (Chisholm, 1993).

All antimicrobial peptides identified so far act stoichiometrically (Boman, 1995). Most are cationic and have hydrophobic, membrane spanning regions, which form ion-channels in the bacterial or cell membrane (reviewed by Boman, 1995). However, some, such as bee apidaecins (Casteels & Tempst, 1994), frog antimicrobial peptides from *Rana* spp. (Park *et al.*, 1994), hemipteran thannatin (Fehlbaum *et al.*, 1996) and mammalian PR-39 (Boman *et al.*, 1993) appear to target specific proteins and do not form pores. Most antimicrobial peptides are bactericidal (Cociancich *et al.*, 1994a; Boman, 1995), but bee apidecins (Casteels *et al.*, 1989) and metalnikowins from the hemipteran, *Palomena prasina* (Chernysh *et al.*, 1996), are bacteriostatic. In addition, some antimicrobial peptides also act against fungi, protozoans, eukaryotic cells or viruses (Cociancich *et al.*, 1994b; Boman, 1995). With regard to viruses, some defensins (Ganz *et al.*, 1985, Daher *et al.*, 1986; Selsted & Harwig, 1987) or bovine antimicrobial peptides (Zerial *et al.*, 1987) inactivate herpex simplex virus 1 *in vitro* , while horseshoe crab tachyplesins are active against several viruses *in vitro* (Morimoto *et al.*, 1991; Murakami *et al.*, 1991).

Table 1.2.3 Examples of the main groups of animal antimicrobial peptides (classification based on Boman, 1995)

Peptide	Properties	Size	Origin	References
Group 1	**Linear, mostly helical, without cysteines**			
Insect cecropins; Mammalian cecropin	Bactericidal (Gram positive and negative). Inducible in insects, constitutive in mammals	4 kDa	Insects (Lepidoptera, Diptera); Mammals (constitutive in pig intestine)	Boman *et al.* (1991); Lee *et al.* (1989)
Magainins; dermaseptins; bombinins	Dermaseptins are antifungal, others bactericidal (Gram positive and negative). Magainins (*Xenopus*) also kill fungi, protozoa. Bombinins-H are hemolytic.	2-2.5 kDa	Amphibians (skin secretions)	Kreil (1994); Barra & Simmaco (1995); Boman (1995)
P-MAP-36; -37; CAP18; hCAP-18	Bactericidal (Gram positive and negative). Constitutive.	2, 4 kDa	Mammalian cathelicidins (neutrophils)	Zanetti *et al.* (1995)
Moricin	Bactericidal (Gram positive and negative). Inducible	5 kDa	Insects (*Bombyx mori*)	Hara & Yamakawa (1995)
Group 2	**Linear, rich in certain residues, without cysteines -proline rich peptides-**			
Bac-5; Bac-7; PR-39; prophenin	Bactericidal (Gram positive and negative), Bac-7 antiviral against HSV-1. Constitutive.	5, 7, & 4 kDa	Mammalian cathelicidins (neutrophils)	Zanetti *et al.* (1995)
Apidaecins	Bacteriostatic (Gram negative). Inducible.	2 kDa	Insects (*Apis mellifera*)	Casteels *et al.* (1989)
Abaecin, lebocins	Bactericidal (Gram positive and negative). Lebocins are o-glycosilated. Inducible	4 & 5.5 kDa	Insects (*Apis mellifera* ;*Bombyx mori*)	Casteels *et al.* (1990); Hara & Yamakawa (1995)
Drosocin; Pyrrhococin, metalnikowins	Drosocin & pyrrhococin, (glycosilated) are bactericidal; metalnikowins bacteriostatic(all Gram negative). Inducible	2 kDa	Insects (*Drosophila melanogaster*), *Pyrrrhococus apterus*)	Bulet *et al.* (1993); Cociancich *et al.* (1994b); Chernysh *et al.* (1995)
Metchnikowins	Bactericidal (Gram positive). Inducible			Levashina *et al.* (1995)

	-Tryptophan rich peptides-			
Indolicidin; PMAP-23	Bactericidal (Gram positive and negative). Constitutive.	ca. 2 kDa	Mammalian cathelicidins (neutrophils)	Zanetti *et al.* (1995)
	-Glycine rich peptides-			
Hymenoptaecin; Coleopterin; Diptericins	Bactericidal (Gram negative). Inducible	10, 8 & 9 kDa	Insects	Cociancich *et al.* (1994a)
Group 3	**Peptides with one disulphide bond**			
Cyclic dodecapeptide	Bactericidal (Gram positive and negative). Constitutive.	<2 kDa	Mammalian cathelicidin (neutrophils)	Romeo *et al.* (1988); Zanetti *et al.* (1995)
Brevinins; ranalexin; esculentins, Thanatin	Bactericidal (Gram positive and negative). Thanatin is fungicidal	2-4 kDa	Amphibians (*Rana* spp.), constitutive (released with mucus), Insects (*Podisus masculiventris*), inducible	Barra & Simmaco (1995); Fehlbaum *et al* (1996)
Group 4	**Two or more disulphide bridges, mainly β-sheets -two disulphide bridges-**			
Tachyplesins, polyhemusins	Bactericidal (Gram positive and negative). Tachyplesins are cytotoxic, possibly antiviral. Constitutive.	2-2.5 kDa	Horseshoe crabs (*Limulus polyphemus, Tachypleus tridentata*). Granular hemocytes.	Iwanaga *et al.* (1994); Morimoto *et al.* (1991); Murakami *et al.* (1991)
Protegrins	Bactericidal (Gram positive and negative). Constitutive.	< 2 kDa	Mammalian cathelicidin (neutrophils)	
	-three disulphide bridges-			
Insect defensins	Bactericidal (Gram positive). Inducible.	4 kDa	Insects (except Lepidoptera), scorpions.	Cociancich *et al.* (1994a)
Defensins	Bactericidal (more against Gram positive), antifungal, antiviral (HSV 1). Constitutive.	4 kDa	Mammals (neutrophils, constitutive)	Selsted & Ouellette (1995)
β-defensins	Bactericidal (more against Gram positive). Constitutive.		Mammals, chicken (neutrophils). Constitutive	Selsted *et al.* (1993); Harwig *et al.* (1994); Selsted & Ouellette (1995)
Limulus big defensin	Bactericidal (more against Gram positive). Constitutive.	8-9 kDa	*L. polyphemus* (granular hemocytes)	Saito *et al.* (1995).

The importance of antibacterial peptides for defence *in vivo* has been investigated in insects (Anderssons *et al.*, 1990; Boman, 1995; Jarosz, 1995). In Lepidoptera, inhibition of cecropin with the anti-cecropin agent of the parasitic nematode *Heterorhabditis bacteriophora*, which harbours symbiotic bacteria, causes death of larve infected with "non-pathogenic" bacteria within 24 h (Jarosz, 1995). Similarly, in *Drosophila* and several species of lepidopterans death occurs after challenge with *E. coli* if mRNA synthesis or translation is blocked with actinomycin or cycloheximide (Boman, 1995). This indicates that antimicrobial peptides play a key role in the rapid response to bacterial infection in insects and presumably other groups (Boman, 1995). However, the importance of antimicrobial peptides for *in vivo* antiviral defense is less clear. In Lepidoptera, challenge of the cabbage looper, *Trichoplusia ni,* with *Autographa california* nuclear polyhydrosis virus does not induce immune proteins, and induction of immune factors by bacterial infection does not afford protection against subsequent infection by the virus (Anderssons *et al.*, 1990). This indicates that, in insects at least, the mechanism of antiviral defense differs from antibacterial or antifungal defence (Anderssons, *et al.*, 1990). Similar work has not, as yet, been carried out in other groups, including the Crustacea.

The sea, with its diversity of invertebrate fauna, has become a prospecting ground for new pharmaceuticals, and whole body homogenates of sessile marine invertebrates have yielded compounds with antibacterial, antiviral or anti-tumor activities, some of which are subject to clinical trials (DeVries & Beart, 1995). Most of these are small (< 1-2 kDa) peptides, many with novel chemical structures, which have not been included in the classification given by Boman (1995). Best known are perhaps the didemnins of the tunicate, *Didemnum* spp. Didemnins have potent activity against cancer cells and against a range of viruses *in vitro* (Rinehardt *et al.*, 1981). The *in vivo* function of most of these compounds is unknown, but it is conceivable that some act in host defence. Thus, halocycamines are tetrapeptide-like antibacterial and antiviral substances which occur in the haemocytes of the solitary

ascidian, *Halocynthia roretzi* (Azumi *et al.*, 1990a; 1990b). Halocycamines have neutralizing activity against the fish virus IPN *in vitro* (Azumi *et al.*, 1990b). Similarly, the morula cells of the solitary tunicate, *C. intestinalis* have recently been found to contain potent activity against Gram positive and Gram negative bacteria (Findlay & Smith, 1995). This activity is due to the presence of several peptides in the molecular weight range of <5 to ca. 10 kDa (C. Findlay, University of St. Andrews, pers. com.). Antibacterial peptides in marine invertebrates are not restricted to deuterostomes, as the anemone, *Actinia equina*, similarly contains an antibacterial peptide of < 5 kDa (D. Hutton, University of St. Andrews, pers. com.).

c) Antibacterial proteins

In addition to the low molecular weight (< 10 kDa) antimicrobial peptides, some larger antibacterial proteins have been described from insects (Cociancich *et al.*, 1994a) and horseshoe crabs (Muta *et al.*, 1987; Iwanaga *et al.*, 1994). Thus, attacins are inducible, glycine rich, bacteriostatic proteins with a molecular mass of 20 kDa which have been isolated from *H. cecropia* (Hultmark *et al.*, 1983) and sarcotoxins II are related bactericidal molecules from the flesh fly *Sarcophaga peregrina* (Ando *et al.*, 1987). In horseshoe crabs, the large granules of the haemocytes contain an 11.6 kDa protein which is released upon exocytosis (Muta *et al.*, 1987; Iwanaga *et al.*, 1994). This protein, termed anti-LPS factor because it inhibits the LPS-mediated activation of the clotting response, is active against Gram negative bacteria (Muta *et al.*, 1987). Neither of these proteins has, as yet, been assessed for antiviral activity.

c) Reactive oxygen species

Reactive oxygen species (ROS) are generated during the respiratory burst (Babior *et al.*, 1973) or as by-products of melanization (Riley *et al.*, 1988; Nappi *et*

al., 1995). Inactivation of microorganisms by ROS produced by the respiratory burst has been described on pages 35-36. Nappi *et al.* (1995) have demonstrated generation of superoxide anion (O_2^-) during melanotic encapsulation in *Drosophila melanogaster in vivo*, but O_2^- or other ROS generated during melanization do not appear to act in the killing of parasites (Nappi *et al.*, 1995). The importance of ROS for antiviral defence in invertebrates thus remains unknown.

Antiviral defence

Antiviral activity has seldom been reported for invertebrates. Tachyplesins (Morimoto *et al.*, 1991; Murakami *et al.*, 1991), halocycamines (Azumi *et al.*, 1990b) and some components derived from whole body homogenates of marine invertebrates (DeVries & Beart, 1995) are known to neutralize viruses *in vitro* (see page 53), but their significance for *in vivo* host defence is unknown. Very little is known about other antiviral factors in invertebrates. Funakoshi & Aizawa (1989) isolated a protease from the gut juice of the silk worm, *Bombyx mori*, which neutralizes nuclear polyhedrosis virus *in vitro*. Antiviral activity *in vitro* has also been detected in the haemolymph of the blue crab, *C. sapidus* (McCumber *et al.*, 1979), the snail, *Biomphalaria glabrata* (Cheng *et al.*, 1983), the oyster, *Crassostrea virginica* (Bachère *et al.*, 1990), and the tobacco budworm, *Heliothis virescens* (Ourth & Renis, 1993), but few antiviral factors have been characterized. The antiviral factor in *C. sapidus* is thought to be an 80 kDa protein (McCumber *et al.*, 1979), whereas in *H. virescens* antiviral activity is linked to the melanization reaction (Ourth & Renis, 1993). In addition, the silkworm, *Bombyx mori*, synthesises a fatty acid-derived factor upon infection with BmNPV, which affords protection against viral infection *in vivo* (Uchida *et al.*, 1984).

In the blue crab, *C. sapidus*, neutralising activity has been detected in the plasma and serum, although activity in the plasma was more pronounced (Mc Cumber *et al.*, 1979). It has not yet been investigated whether or not neutralising factors are present in the granular haemocytes of crustaceans, which contain antibacterial factors (Chisholm & Smith, 1992; 1995), or in other tissue extracts, or gut juice. A main shortcoming for any study of antiviral defence in crustaceans is the lack of suitable cell lines to propagate crustacean viruses (Nadala *et al.*, 1993). Until these become available, bacteriophages or viruses which can be propagated on established vertebrate or invertebrate cell lines must be used as test particles. Bacteriophages are dissimilar to animal viruses and viruses generally do not have shared antigens, such as LPS, peptidoglycans or β-1,3 glucans, present in bacteria or fungi. However, the finding that some crustaceans can clear bacteriophages from the haemolymph (Taylor *et al.*, 1964; McCumber & Clem, 1977) and neutralize them *in vitro* (McCumber *et al.*, 1979) indicates that these viruses are suitable for a study on antiviral defence in crustaceans.

Aims of the present study

Viral diseases are increasingly prevalent in crustacean aquaculture (Fulks & Main, 1992; Hassan *et al.*, 1995; Inouye *et al.*, 1996; see Table 1.1.5, pages 19-20). In addition, viruses derived from sewage pollution may be transmitted to humans by commercially exploited species (diGirolamo, 1992) and crustaceans may serve as vectors for fish viruses, which could harm both aquaculture and fisheries (Halder & Ahne, 1988; Mortensen, 1993; Mortensen *et al.*, 1993). An understanding of antiviral defence in crustaceans is therefore important, yet from the above review it is apparent that we know very little about this response.

It is proposed that adult crustaceans do possess antiviral defences, because viral diseases appear to affect primarily larvae or stressed animals (Mari, 1987;

Lightner, 1988; Overstreet *et al.*, 1988; Leblanc & Overstreet, 1990). Using the shore crab, *Carcinus maenas*, as experimental animal, this study will therefore address the following aims:

1. To ascertain whether or not *C. maenas* is capable of recognizing and clearing viral particles *in vivo* and whether the clearance of viral particles is mediated by haemocytes;

2. To determine whether or not viral particles activate proPO *in vitro*. Phenoloxidase may be important in the neutralisation of viruses or may serve as a marker for the activation of the proPO cascade which may include antiviral factors;

3. To examine haemolymph extracts and tissue extracts of *C. maenas* for neutralising activity against viruses;

4. To characterize the antibacterial factors in *C. maenas* haemocytes (Chisholm & Smith, 1992) and investigate whether or not these factors act in antiviral defence.

2. ANTIVIRAL DEFENCE IN THE SHORE CRAB, *CARCINUS MAENAS*

2.1 CLEARANCE OF INJECTED BACTERIOPHAGES FROM THE HAEMOLYMPH OF *C. MAENAS*

Introduction

A prerequisite of host defence is the ability to recognize foreign particles and encapsulate them or eliminate them from the body. The ability of crustaceans to clear injected viral particles from the haemolymph has been studied by Taylor *et al.* (1964) in *C. maenas* and by McCumber & Clem (1977) in the blue crab, *Callinectes sapidus*. Taylor *et al.* (1964) found that the coliphage T_1 is eliminated more rapidly from the haemolymph of *C. maenas* after a secondary injection, following clearance of a primary challenge. However, this study was based on only two crabs and did not consider clearance within the first two weeks post injection (Taylor *et al.*, 1964). A more detailed study was subsequently carried out by McCumber & Clem (1977) on *C. sapidus*, using a range of radiolabelled viruses and non-self proteins. These authors found that *C. sapidus* is capable of totally eliminating injected coliphage T_2 from the haemolymph within 30 minutes, and is able to reduce the titres of injected T_4 phage or poliovirus within 3 h (McCumber & Clem, 1977). A range of other bacteriophages were not cleared from the haemolymph (McCumber & Clem, 1977). Whilst this work indicates that *C. sapidus* can discriminate between different viruses, irrespective of their size (McCumber & Clem, 1977), it does not explain the mechanism of viral clearance. It is known that crustaceans are capable of clearing a range of injected substances from the haemolymph in addition to viruses, such as bacteria (Tyson & Jenkin, 1973; Smith & Ratcliffe, 1980b; White & Ratcliffe, 1982), non-self proteins (Teague & Friou, 1964; Stewart & Foley, 1969; Sloan *et al.*, 1975; McCumber & Clem; 1977, Mullainadhan & Ravindranath, 1984), or dyes (Fontaine & Lightner, 1974; Mullainadhan *et al.*, 1984). In some cases, this response is associated with lodging of haemocyte aggregates in the gills (Fontaine & Lightner, 1974; Smith & Ratcliffe, 1980b; White & Ratcliffe, 1982, Mullainadhan *et al.*, 1984). In *C. maenas* which are given injections of Gram positive or Gram negative marine bacteria, the appearance of these aggregates is preceeded by a decrease in the number of circulating haemocytes (Smith & Ratcliffe, 1980a; White & Ratcliffe,

1982). In the present chapter, work was conducted to confirm that *C. maenas* is capable of recognizing and removing a range of viral particles from the haemolymph and to study the kinetics of this response within the first 14 days. It was also investigated whether viral clearance is haemocyte-mediated.

Bacteriophages were chosen as convenient test particles, because they can be easily propagated and purified and do not pose a health hazard to vertebrates or invertebrates. The phages selected for this study were the coliphages T_2 and T_4, used by McCumber *et al.* (1977) in the clearance study of *C. sapidus*, and the marine *Pseudomonas* phage Φ-111, which occurs in the waters around Scotland and may be encountered by *C. maenas* in its natural environment.

Materials and Methods

Animals

Specimens of *C. maenas* were collected in baited creels from St. Andrews Bay and maintained in filtered, aerated seawater (salinity 32‰ ± 3‰), at 10-15°C, for no longer than two weeks prior to use. The crabs were fed twice weekly on chopped herring. Only healthy male specimens of *C. maenas* with carapace width 6.93 ± 0.09 cm, weight 85.50 ± 2.91 g (in a sample of 16 animals) were selected for clearance studies.

Propagation of bacteriophages

Coliphage T_2 (NCIMB 10358) was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). Coliphage T_4 and it's host, *E. coli* strain C 600, was gifted by Prof. W.C. Russell, University of St. Andrews. *E.*

coli strain D 22 (CGSC 5163) was obtained from the *E. coli* Genetic Stock Center (Yale University, USA). The marine bacteriophage Φ-111 and its host, the Gram negative bacterium, *Pseudomonas* 111, were gifted by Dr. Birkbeck, University of Glasgow. The phages were maintained in suspension or on plate cultures at 4°C. *E. coli* strains were maintained on slopes of Lab Lemco agar (Oxoid, Basingstoke, Hampshire) or Luria Bertani (LB) agar (Fluka, Gillingham, Dorset) at 4°C. *Pseudomonas* 111 was maintained on marine agar slopes (Difco, Michigan, Detroit) at 4°C.

Propagation of the T-phages was modified from the protocol for phage λ given in Maniatis *et al.* (1982). The host bacteria, *E. coli* C 600 or *E. coli* D 22, were grown overnight in Lab Lemco broth (Oxoid) or LB broth (Fluka) at 37°C. The bacteria were harvested by centrifugation at 2000 *g* for 10 min at 18°C, washed once in sterile phosphate buffered saline (PBS) (Oxoid) and re-suspended in sterile PBS to an absorbance of 1.0 at 600 nm. This gives a concentration of ca. 1 x 10^9 colony forming units (cfu) ml^{-1}. A suspension of T_2 or T_4 was diluted in salt-magnesium buffer (SM buffer: 0.1 M NaCl, 12 mM $MgSO_4 \cdot 7\ H_2O$, 0.05 M tris, 0.01% gelatin, pH 7.5) (Maniatis *et al.*, 1982) to give ca. 1 x 10^8 plaque forming units (pfu) ml^{-1}. One hundred microlitres of phage suspension was added to 900 µl of the bacterial suspension and incubated on an orbital shaker for 20 min at 18°C to allow the phages to absorb to the hosts. The mixture was then added to 50 ml of nutrient broth, pre-warmed to 37°C, and incubated for 16 h at 37°C until lysis of the host was complete.

For propagation of the Φ-111 phage, it's host, the marine bacterium, *Pseudomonas* 111, was grown for 30 h at 18°C in marine broth (Difco) containing a final concentration of 0.01% agar (Oxoid). Stock phage Φ-111 was diluted in marine SM buffer (MSM: 0.45 M NaCl, 12 mM $MgSO_4 \cdot 7\ H_2O$, 0.05 M tris, 0.01% gelatin, pH 7.5) to give a concentration of ca. 1 x 10^7pfu ml^{-1}. Ten microlitres of the phage

suspension were added to each of five 100 µl aliquots of the *Pseudomonas* 111 suspension taken directly from the broth culture. The phage-host mixtures were incubated on an orbital shaker for 20 min at 18°C to allow the phage to absorb to the host. Each suspension was then mixed with 3 ml of sterile 1/2 strength marine agar (Difco) in MSM buffer, at 45°C, and poured onto marine agar (Difco) plates. The plates were incubated for 16 h at 25°C to allow plaques to develop. Subsequently, 5 ml MSM buffer were added to each plate and they were incubated on an orbital shaker at 18°C for a further 2 h to allow the phages to diffuse into the buffer. Following incubation, the buffer from each plate was aspirated and pooled, together with 1 ml of MSM buffer used for washing each plate.

Purification of bacteriophages.

The protocol for the purification of phages was modified from Maniatis *et al.* (1982). One millilitre of chloroform (Sigma, Poole, Dorset) was added to each phage or host (control) suspension and mixed briefly to lyse remaining bacterial cells. Pancreatic RNAse and DNAse (Sigma) were then added to final concentrations of 10 µg ml^{-1} to digest bacterial nucleic acids which may trap phage particles. The suspensions were incubated for 2 h at 18°C and centrifuged at 2000 *g* for 5 min. Then NaCl was added to each supernatant to give final concentrations of 1 M. The supernatants were incubated on ice for 1 h and re-centrifuged at 11, 000 *g* for 10 min at 4°C. Solid PEG 8000 (8 g 100 ml^{-1} w/v) was added to the supernatants and the suspensions were incubated overnight at 4°C. After re-centrifugation at 11 000 *g* for 10 min, the supernatants were discarded into bleach (final concentration of 5 ml l^{-1}) and the tubes were drained onto tissue paper to remove any remaining fluid. The tubes were then washed with 1 ml of SM buffer for T-phages, or MSM buffer for Φ-111, to resuspend the phages and the suspensions transfered to 15 ml polypropylene tubes (Sterilin, Stone, Staff.). An equal volume of chloroform (Sigma) was added and the suspensions vortexed for 30 s. They were finally centrifuged at 2000 *g* for

10 min at 4 °C. The aqueous phase was recovered and the chloroform extraction step was repeated. The purified phages or control suspension were stored at 4°C for up to six months without appreciable change in phage titre.

Titration of Bacteriophages

The T-phages were ten fold serially diluted to 10^{-8} in SM buffer. Three aliquots of 100 µl from each dilution were added to 100 µl each of a log-phase host culture, grown as described above, and incubated on an orbital shaker for 20 min at 18°C. The phage-host suspension was then mixed with 3 ml of sterile 1/2 strength Lab Lemco agar (Oxoid) or LB agar (Fluka) at 45°C and poured onto Lab Lemco (Oxoid) or LB (Fluka) agar plates. The phage overlays were incubated for 16 h at 37°C. Plaques were counted and the phage titres expressed as the number of plaque forming units (pfu) ml^{-1}. The protocol for the titration of the phage Φ-111 was the same as for the T-phages, except that the phage-host suspension was mixed with 3 ml of 1/2 strength marine agar (Difco) in MSM buffer at 45°C, poured onto marine agar (Difco) plates and the overlays incubated for 16 h at 25°C.

Phages for use in experiments were diluted ten-fold in sterile *Carcinus* saline (CS) (0.45 M NaCl, 13 mM KCl, 30 mM $CaCl_2 \cdot 6\ H_2O$, 0.26 M $MgCl_2 \cdot 6\ H_2O$, 0.05 M tris, pH 7.4) (modified from Smith & Ratcliffe, 1978) to give final concentrations of 2-4 x 10^9 pfu ml^{-1}.

Treatment of crabs and preparation of samples

One hundred microlitres of phage suspension were injected into the unsclerotised membrane at the base of the carapace of each experimental crab. Control crabs were left untreated. For haemocyte enumeration experiments (see below), two further control groups received an injection of 100 µl of a similarly

prepared extract from an uninfected culture of *E. coli* C 600, or 100 µl of CS. All inoculations for the phage clearance experiments (see below) were carried out between 10 am and 12 pm and for the haemocyte enumeration experiments at 12 pm. At specified times post-injection (see below), the unsclerotized membrane of the right cheliped of each crab was sterilized by swabbing with ethanol and ca. 0.5 ml haemolymph were withdrawn using pre-chilled 19 gauge needles and a 2.5 ml syringes. For phage clearance experiments, the haemolymph was ten-fold serially diluted in ice-cold sterile CS to between 10^{-3} or 10^{-5}. For haemocyte counts, the haemolymph was diluted ten-fold in ice-cold marine anticoagulant (0.45 M NaCl, 0. 10 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA) (Söderhäll & Smith, 1981), pre-sterilised by passing through a 0.22 µm filter membrane (Millipore, Watford, UK).

Clearance experiments

To establish whether or not *C. maenas* is able to clear bacteriophages from the haemolymph, four groups of 4-8 crabs received inocula of 2-4 x 10^8 pfu of T_2, T_4 or Φ-111 in 100 µl sterile CS, and were bled after 0.25 h, 0.5 h, 1.5 h, 3 h or 6 h. Phages were counted as described above.

To determine the retention time of bacteriophages in the haemolymph, eight groups of 5-8 crabs were given injections of ca. 3 x 10^8 pfu of T_2 in 100 µl sterile CS, and bled at 0.5 h, 3 h, 6 h, 24 h, 48 h, 72 h, 168 h or 336 h post injection. Phages were enumerated as above.

To investigate the dose-dependent clearance of bacteriophages, four groups of 4-9 crabs each received an injection of 100 µl of 10^8, 10^7, 10^6 or 10^5 pfu of T_2 in 100 µl sterile CS. Haemolymph was withdrawn at 3 h post infection, diluted and the phages enumerated as above.

To ascertain the effect of a primary injection on clearance of bacteriophages from the haemolymph, two groups of three crabs were given a primary injection of ca. 2 x 10^8 pfu of T_2 and a secondary challenge of ca. 2 x 10^8 pfu of T_2, both in 100 μl sterile CS, after either 168 h or 72 h. A further group of three crabs which had not received a primary injection was also given an inoculation of ca. 2 x 10^8 pfu of T_2. Haemolymph was withdrawn at 0.5 h, 3 h, 6 h and 24 h and assayed for phages as above.

Haemocyte counts

To determine the effect of bacteriophage T_2 on haemocyte counts, four groups of 4-7 crabs were given 100 μl injections of T_2, ten-fold diluted in sterile CS to contain a final concentration of ca. 2 x 10^7 pfu. Two control groups were given 100 μl injections of sterile CS or of an extract of *E. coli* C 600, purified as described for the T-phages and diluted as above. An additional control group was left untreated. Crabs were bled after 0.5 h, 3 h, 6 h or 24 h and the haemolymph was diluted immediately ten-fold in sterile MA. The haemocytes were counted with an Improved Neubauer Haemocytometer and cell counts expressed as haemocytes ml^{-1}.

Fate of bacteriophage T_2

To determine the fate of injected bacteriophage, seven groups of 3-4 crabs were given inoculations of ca. 3 x 10^8 pfu of T_2 in 100 μl sterile CS. A control group of 3 crabs was left untreated. At 0.5 h, 3 h, 6 h, 24 h or 48 h post injection, 0.5 ml haemolymph was withdrawn and serially ten-fold diluted with sterile CS to 10^{-4}. The crabs were surface sterilized by swabbing with ethanol and subsequently killed by removing the upper part of the carapace and severing the central ganglion which is located beneath the heart, with sterile scissors. Subsequently, 0.1 g (wet weight) of

the hepatopancreas, heart or gill were removed with a sterile scalpel and sterile forceps. These tissues are known to contain fixed phagocytes in the related species *Callinectes sapidus* (Johnson, 1991). The tissue samples were suspended in 1 ml sterile SM buffer, homogenized for 5 min on ice with a sterile 1 ml glass piston homogenizer, and centrifuged at 2000 g for 5 min at 4°C. The supernatant was diluted and the phages were titrated as described above.

Statistical analysis

The clearance of bacteriophage T_2 was analyzed by two-fold segmented linear regression (or piecewise regression) of log pfu against time post injection. Piecewise regression is used to analyse functions with distince phases which can be described by two or more successive linear regressions (Neter *et al.*, 1985; Lehman *et al.*, 1994). The dose-response of phage clearance was analyzed by linear regression of log pfu at 3 h against log dose (Lehman *et al.*, 1994) as described in Neter *et al.* (1985). Haemocyte counts were compared by one factor ANOVA (Zar, 1984) of treatments at each time of bleeding. A probability of $P<0.05$ was accepted as significant.

Results

Clearance experiments

Figure 2.1.1 shows that between 80-90 % of coliphages T_2 or T_4 were cleared from the circulation of *C. maenas* within 6 h post injection (Figure 2.1.1). By contrast, the marine *Pseudomonas* phage Φ-111 was not cleared. The titre of Φ-111 remained constant at around 6.5 log units ml^{-1} (Figure 2.1.1), whereas the titre of T_2 was reduced by at least 82.3 % after 3 h and by a further 43.1 % after 6 h, indicating

that the rate of clearance was not constant and faster during the initial phase (Figure 2.1.1). In the case of the T_4 phage, the titre was reduced by at least 63.5 % after 3 h and by 54.9 % after 6 h, indicating an initially somewhat faster, but overall closer to exponential, rate of clearance (Figure 2.1.1).

Figure 2.1.2 shows the clearance of T_2 from the circulation of *C. maenas* over a period of two weeks, clearly indicating that the initial rapid decrease is followed by a slower decrease in phage titre, but T_2 persisted in the haemolymph of *C. maenas* for at least two weeks after injection (Figure 2.1.2). The inconstant clearance rate of phage T_2 from the circulation of *C. maenas* over a period of 24 h is analyzed in Figure 2.1.3. The clearance curve (Figure 2.1.3) follows the two-fold segmented linear regression model described by Lehmann *et al.* (1994). The closest fit of the model is achieved with an intersection point at 3 h post injection, indicating a significant change in clearance rate at this point (Figure 2.1.3). The segmented regression given in Figure 2.1.3 is highly significant ($P<0.001$).

A regression of the concentration of the injected phage (dose) versus pfu ml^{-1} in the haemolymph of *C. maenas* at 3 h post injection is shown in Figure 2.1.4. The regression is highly significant ($P < 0.001$), indicating that the extent of clearance of T_2 phage from the circulation of *C. maenas* over this time depends only on the injected dose and follows the same rate, irrespective of the injected dose (Figure 2.1.4).

Figure 2.1.5 shows the clearance of T_2 from the haemolymph of *C. maenas* previously injected with T_2 at 72 h or 168 h. The phage titre in crabs which had received an injection of T_2 at 72 h or 168 h, did not decrease faster or slower than that of crabs which had received no previous injection, implying that clearance was not enhanced or reduced by prior challenge with phage (Figure 2.1.5).

Haemocyte counts

Haemocyte counts of *C. maenas* which had received an injection of T_2, or a control injection at 0.5 h, 3 h, 6 h, or 24 h previously, or of untreated crabs, are shown in Figure 2.1.6. Significant changes in total haemocyte number were not observed with any of the treatments (Figure 2.1.6). Similarly, significant changes in differential haemocyte numbers (granular cells, semigranular cells or hyaline cells) were not apparent.

Fate of bacteriophage T_2

Figure 2.1.7 shows the distribution of T_2 in the haemolymph, heptopancreas, heart or gills of *C. maenas* at 0.5 h, 3 h, 6 h, 24 h or 48 h post injection. A rapid decline of T_2 titres from the haemolymph (overall clearance 95.2% over 48 h) is mirrored by a rapid increase of phage titre in the hepatopancreas within 6 h post injection (Figure 2.1.7). Phage titres in the hepatopancreas reach a maximum at 6 hours post injection, followed by a slow decline (Figure 2.1.7). The phage count in the hepatopancreas at 48 h is 25.2 % of the maximum count at 6 h (Figure 2.1.7), but persists virtually unchanged from 48 h to 72 h post injection, when it is 22.6% of the maxiumum count (Figure 2.1.7, legend). Phage numbers in the heart and gill remain stable around 5 log units (Figure 2.1.7).

Discussion

The data presented in this chapter show that *C. maenas* is capable of clearing the bacteriophages T_2 and T_4 from the haemolymph, but not apparently the marine phage Φ-111. The initial clearance of the T_2 phage was rapid, but was followed by a slower rate of removal, which could best be analysed by a segmented linear regression (Lehmann *et al.,* 1994). The phage persisted in the circulation for at least two weeks. Clearance rates of T_2 were dose independent and not affected by pre-challenge with phages at three days or one week prior to secondary injection.

Injection of T_2 or controls did not result in changed haemocyte numbers when compared to untreated crabs. The phage was sequestered to the hepatopancreas within 6 h post injection, where it persisted at high titres for at least 72 h.

These results indicate that *C. maenas* is capable of recognizing injected viral particles as foreign and can discriminate between different viruses. However, it remains unclear whether or not this discriminative ability is attributable to the size, shape or general surface properties of the injected phages. For the blue crab, *C.allinectes sapidus*, McCumber and Clem (1977) found that differential clearance of bacteriophages or poliovirus occur independently of viral size and are therefore not due to simple filtration. However, size-based filtration ('sieving') is only one mechanism of particle retention (LaBarbera, 1984). Other properties of particles, such as charge or hydrophobicity, determine the efficiency with which they adhere to surfaces (LaBarbera, 1984). In insects, Lackie (1983) showed that charge and hydrophobicity are important determinants of phagocytosis and encapsulation of non-self particles. Alternatively, virus clearance may be mediated by humoral or cellular 'receptors' (recognition molecules). The presence of a humoral 'receptor' for T_2 in the plasma of the blue crab, *C. sapidus*, has subsequently been reported by McCumber *et al.* (1979). Similarly, 'receptors' for the phages T_4 and T_7 have been found in the serum of the snail *Biomphalaria glabrata* by Cheng *et al.* (1983). These respective 'receptors' neutralized the phages (McCumber *et al.*, 1979; Cheng *et al.*, 1983). However, using radiolabelled T_2 phage, McCumber & Clem (1979) were able to demonstrate that phage clearance was not solely attributable to neutralising activity. Similarly, neutralisation of phages was not found to be a preerequisite for clearance in insects (Berheimer *et al.*, 1952) or oysters (Feng, 1966). At present, it is not known whether or not similar 'receptors' are present in the haemolymph of *C. maenas* or if *C. maenas* is capable of neutralising bacteriophages *in vitro*.

One way to determine the presence of 'receptors' is to determine whether or not clearance is slowed or prevented by the injection of higher doses of material. In the crayfish, *Procambarus clarkii* (Sloan *et al.*, 1975) and the blue crab, *C. sapidus*, (McCumber & Clem, 1977), the clearance of injected bovine serum albumin (BSA) can be retarded by injecting milligram quantities of unlabelled BSA together with microgram quantities of radiolabelled BSA. This retardation is not evident after injection of a similar mixture of unlabelled bovine gamma globulin and labelled BSA, indicating that these animals possess specific 'receptors' for these proteins (Sloan *et al.*, 1975; McCumber & Clem (1977). In *C. sapidus* , these 'receptors' are not located in the haemolymph (Clem *et al.*, 1984). In the present study, the injection of higher doses of T_2 did not lead to slower clearance of T_2 from the haemolymph of *C. maenas*, as would result from the saturation of specific 'receptors'. However, it is possible that the highest dose of injected phage (ca. 2×10^8 pfu) was insufficient to bring about saturation of the putative 'receptors', as this amount represents only nanogram quantities of material (Garen & Kozloff, 1959).

In contrast to McCumber & Clem's (1977) findings for the clearance of T_2 from the haemolymph of *C. sapidus* , the T_2 phage was not completely eliminated from the circulation of *C. maenas*. The rate of clearance was inconstant, rather than exponential, and faster during the initial phase. This response is best described by a linear regression which is segmented at 3 h post injection. Segmented linear regression is used for analysis of linear functions with distinct phases (Neter, 1985). Alhough a distinct cut-off at 3 h post injection is unlikely, this method offers the best description of clearance kinetics of T_2 from the haemolymph of *C. maenas*. The same model was used by Lehmann *et al.* (1994) in a study on the clearance of *Onchocercia liennalis* microfiliaria from the haemolymph of the blackfly, *Simulium vittatum.* The presence of a distinct phase of more rapid initial clearance can be due to several reasons. First, the clearance rate may slow down because of the saturation of specific "receptors" (Sloan *et al.*, 1975; McCumber & Clem, 1977) or otherwise

interacting surfaces. Second, remaining particles may become coated and thereby gain protection from clearance factors which can no longer distinguish them as foreign (Lehmann *et al*, 1994) or lose affinity to surfaces. Third, the collision rate between particles and clearance factors, required for an escalated clearance response, decreases due to their relative depletion, as has been described for the clearance of injected bacteria in insects by Gagen & Ratcliffe (1976). The first of these explanations is unlikely, because the clearance rate of T_2 from the circulation of *C. maenas* is dose independent. The second explanation is possible, but it is at present unknown whether or not bacteriophages can bind to, and become coated with, self components of *C. maenas*. With regard to the third, the clearance of bacteria from the haemolymph of *C. maenas* (Smith & Ratcliffe, 1980b) follows similar kinetics to that observed for the clearance of T_2 in the present chapter, in that rapid initial clearance of injected bacteria is followed by a slower decline. Injection of bacteria into the haemolymph of *C. maenas* is associated with a rapid drop in total haemocyte counts and in the counts of granular or hyaline haemocytes (Smith & Ratcliffe, 1980b). However, in the present study, the injection of T_2 phage did not lead to a change in total or differential haemocyte numbers in *C. maenas*. At present, the reason for the more rapid initial clearance of T_2 from the haemolymph of *C. maenas* remains unknown.

Although in the present study, no decline in haemocyte numbers has been observed in *C. maenas* after injection of bacteriophages, it is possible that haemocytes mediate defence against pathogenic viruses in arthropods. Some previous studies have shown that haemocyte counts decline in virus-infected *C. maenas*, although changes in counts do not occur until several days post infection and do not lead to complete clearance of infectious virus (Bang, 1971; Bang, 1974; Hoover & Bang, 1978). Declines in haemocyte counts have also been observed in virus-infected insects (Shapiro 1967; 1968; Shapiro *et al.*, 1969; Davies *et al.*, 1987; Andersons *et al.*, 1990, Miranpuri *et al.*, 1992). Shapiro (1967; 1968) and Shapiro *et*

al. (1969) observed decreases in haemocyte numbers in larvae of the moths *Galleria mellonella* or *Heliothis zea*, respectively, which have been infected with nuclear polyhydrosis virus (NPV). However, these authors concluded that haemocyte drops could have resulted from starvation and stress of infected animals (Shapiro 1967; 1968; Shapiro *et al.*, 1969). Davies *et al.* (1987) reported that the inhibition of melanin formation and of encapsulation of parasitoid eggs in larvae of the tobacco budworm, *Heliothis virescens* infected with *Campoletes sonorensis* polydnavirus is due to a specific decrease in the number of plasmatocytes, caused by the virus. A decrease in melanin formation, together with a decrease in haemocyte numbers, was also observed by Andersons *et al.* (1990) in NPV-infected larvae of the cabbage looper, *Trichoplysia ni*. However, in either of these studies, the decreases may have resulted from infection of the haemocytes themselves (Davies *et al.*, 1987; Andersons *et al.*, 1990). Conversely in nymphs of the grasshopper, *Melanoplus sanguinipes*, infected with an entomopoxvirus, Miranpuri *et al.* (1992) did not observe a decline of haemocyte numbers until late in infection, when the development and growth of diseased animals was significantly retarded. Therefore, in the case of virus infected insects, it remains unknown whether the decrease in haemocyte numbers is a defense response or a result of infection (Shapiro 1967; 1968; Shapiro *et al.*, 1969; Davies *et al.*, 1987; Andersons *et al.*, 1990; Miranpuri *et al.*, 1992). However, in *C. maenas*, encapsulation of infected tissues by haemocytes was observed, and it is possible that this is an *in vivo* defence response against viral infection (Hoover & Bang, 1978). Significantly, all these responses were observed after infection by specific pathogenic viruses. If a haemocyte-mediated defence response is directed at infected tissues rather than at the virus itself, bacteriophages, which cannot infect eukaryotic organisms, are inadequate models for the study of *in vivo* antiviral defence in *C. maenas*.

Despite the absence of antibodies in invertebrates (e.g. Stewart, 1992), several authors have investigated whether or not crustaceans mount an increased

response to a secondary injection of non-self components, following a previous challenge (Taylor *et al.*, 1964; Mullainadhan & Ravindranath, 1984; Clem *et al.*, 1984). In particular, Taylor *et al.* (1964) showed that *C. maenas* clears injected T_1 phage more rapidly after a primary injection 42-70 days prior to a secondary challenge. However, this study was based on only two crabs (Taylor *et al.*, 1964). In a later study, Mullainadhan & Ravindranath (1984) demonstrated a significantly higher clearance rate of injected horseradish peroxidase, but not haemoglobin, from the haemolymph of the mud crab, *Scylla serrata,* after repeated injections of either protein over 48 h. Conversely, Clem *et al.* (1984) did not detect enhanced clearance of injected BSA from the haemolymph of *C. sapidus* which had received a primary injection of BSA 3 days or 10 days previously. Similarly, in the present study, no enhancement of clearance of T_2 by *C. maenas* was evident after pre-challenge at 72 h or 168 h.

In conclusion, although the titres of T_2 or T_4 phages in the haemolymph of *C. maenas* are reduced rapidly, low titres of T_2 persist in the haemolymph for at least two weeks and T_2 remains viable in tissues for at least three days. Because the hepatopancreas is the main site for viral infections in crustaceans (Johnson & Lightner, 1988) and because the T_2 phage was sequestered to this organ, the role of the clearance response in antiviral defense of *C. maenas* remains unclear. The persistance of at least some phages in crabs for lengthy periods is of concern for fisheries and aquaculture, as commercially exploited crustaceans may be exposed to potentially pathogenic human enteric viruses in sewage-polluted waters (Hejkal & Gerba, 1982). Hejkal & Gerba (1982) showed that *C. sapidus* can take up poliovirus from contaminated water, although the virus is not concentrated in the crabs, and DiGirolamo *et al.* (1972b) showed that the edible crabs, *Cancer magister* and *Cancer attennaris,* can take up the bacteriophage T_4 from contaminated water and food and that this phage persists in the crabs for at least 48 h.

Whereas passive filtration of charged or hydrophobic phage particles by the hepatopancreas cannot be ruled out as mechanism of phage clearance in *C. maenas*, haemocyte-mediated recognition remains a possibility. Because the proPO cascade represents the main recognition pathway in the crustaceans (e.g. Smith & Söderhäll, 1986; Söderhäll, 1994; Smith, 1996), proPO activation could serve as a convenient marker to determine whether or not viruses are recognized by the haemocytes. The *in vitro* activation of proPO by viruses was therefore investigated in the following chapter.

Figure 2.1.1 Clearance of injected bacteriophages Φ-111, T_2 or T_4 from the haemolymph of *C. maenas*. Crabs received inocula of 2-4 x 10^8 pfu of phages. At the times indicated in the graph, ca. 0,5 ml haemolymph was withdrawn, diluted in ice-cold CS and the phages were enumerated as described in Materials and Methods. Four to eight crabs were used for each treatment at each time point.

Figure 2.1.1

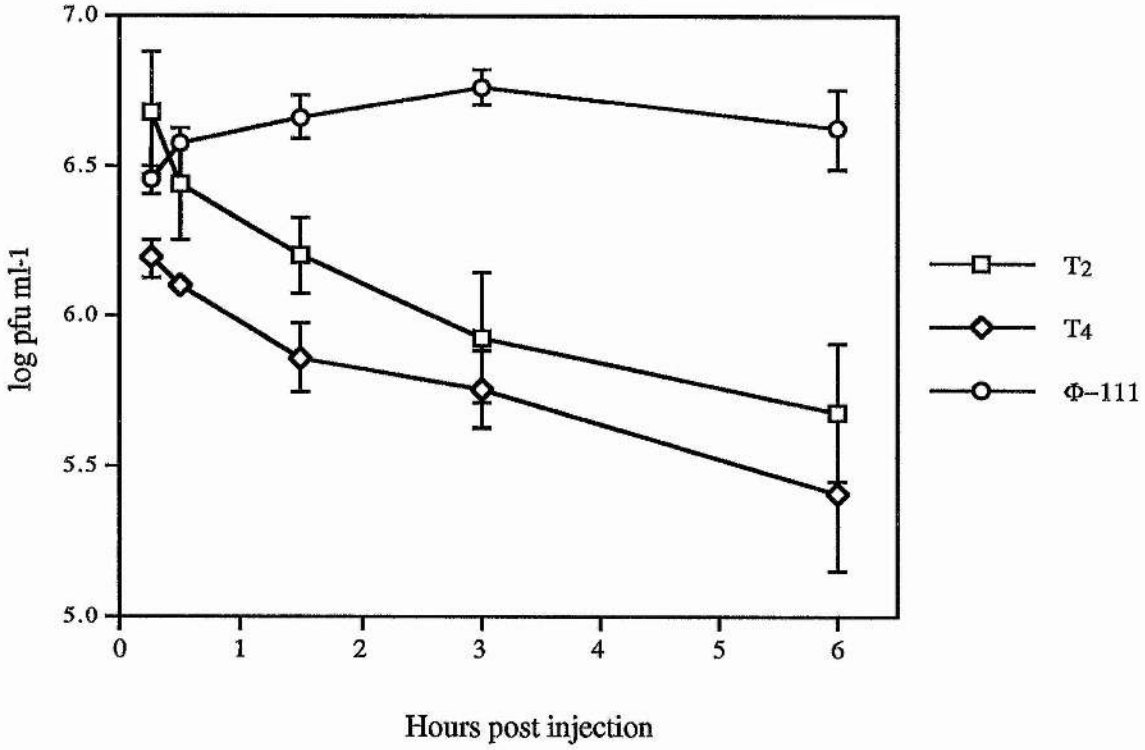

7.0
6.5
6.0
5.5
5.0
log pfu ml-1
0
1
2
3
4
5
6
Hours post injection
T2
T4
Φ-111

Figure 2.1.2 Clearance of injected bacteriophage T_2 from the haemolymph of *C. maenas* over a period of two weeks. Crabs received injections of ca. 2 x 10^8 pfu of T_2. Injections, bleeding and phage enumeration were carried out as described in Materials and Methods. Between five and eight crabs were treated at each time point.

Figure 2.1.3 Two-fold segmented linear regression of clearance of injected T_2 clearance from the haemolymph of *C. maenas* . Data are as for Figure 2.1.2, excluding time-points after 24 h. The intersect at 3 h post injection represents the best fit for the model with $P < 0.001$.

Figure 2.1.2

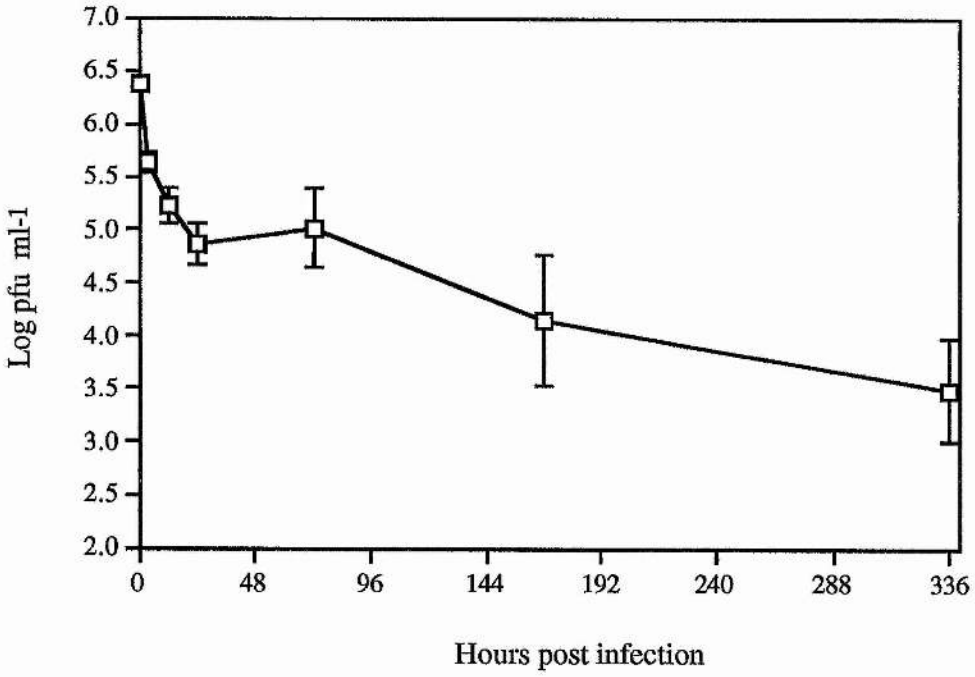


Figure 2.1.3

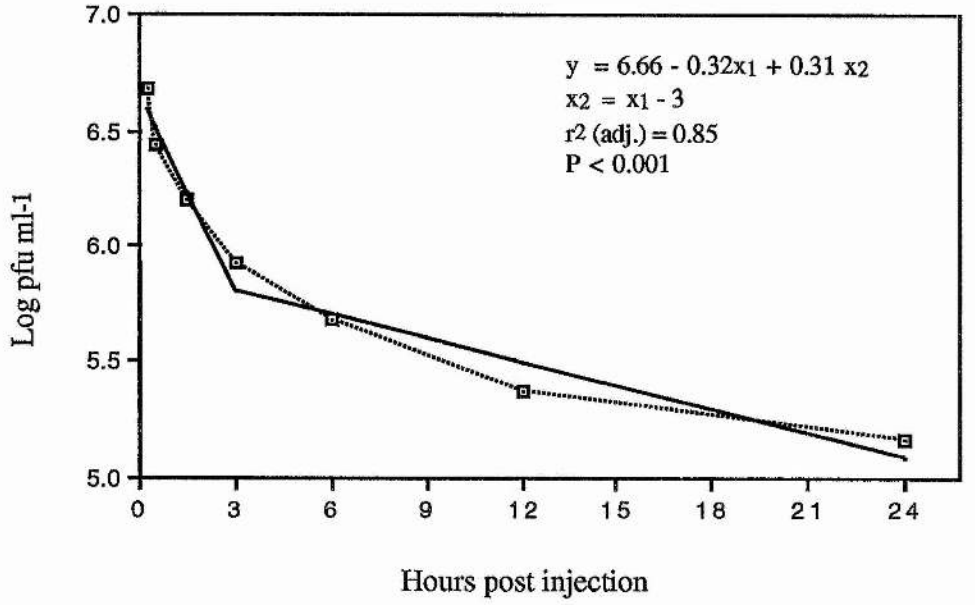


Figure 2.1.4 Linear regression of T_2 clearance from the circulation of *C. maenas* at 3 h post injection with increasing injected dose. Crabs were given ca. 2×10^8, 2×10^7, 2×10^6 or 2×10^5 pfu ml^{-1}. Bleeding and phage enumeration were carried out as described in Material and Methods. Points on the graph represent mean counts for individual crabs ± SE. Between four and seven crabs were used for each dose.

Figure 2.1.4

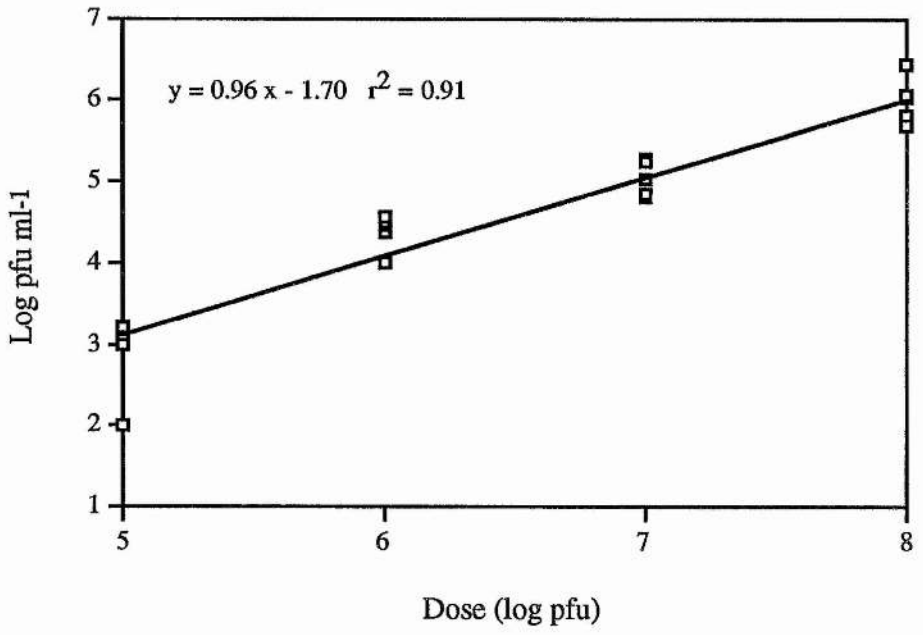


Figure 2.1.5 Clearance of T_2 from the haemolymph of *C. maenas* given primary injections of ca. 1 x 10^8 T_2 at 72 h or 168 h prior to the experiment. Control animals were not challenged. Injections, bleedings and phage enumeration were carried out as described in Materials and Methods. In pre-challenged crabs, the count of T_2 prior to re-inocculation was 5.31 $\pm$ 0.05 log units ml^{-1} in the haemolymph of crabs injected 72 h previously and 2.58 $\pm$ 01.46 log units ml^{-1} in the haemolymph of crabs injected 168 h previously. These values represent ca. 10.8% and 0.01% of the 3 h count, respectively.

Figure 2.1.5

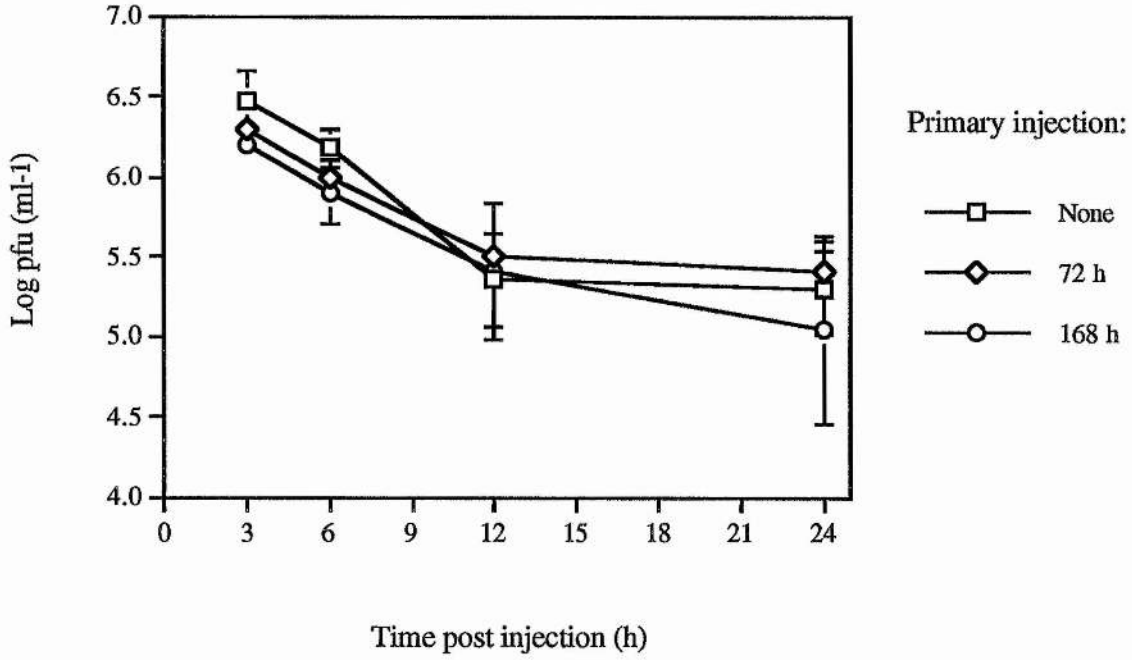

7.0
6.5
6.0
5.5
5.0
4.5
4.0
Log pfu (ml-1)
0
3
6
9
12
15
18
21
24
Time post injection (h)
Primary injection:
None
72 h
168 h

Figure 2.1.6 Total haemocyte counts in *C. maenas* at different times after injection with bacteriophage T_2 or controls. Treatments were as follows:

a) T_2 (ca. 1 x 10^7 pfu in 100 µl sterile CS);
b) C 600 extract (100 µl);
c) CS (100 µl);
d) untreated.

The T_2 stock or C 600 extract, which was prepared in the same manner as the phage stock, were diluted ten fold, to decrease residual bacterial endotoxins which may affect haemocyte counts. Crabs were bled and haemocytes enumerated as described in Materials and Methods. Values represent means ± SE of averaged duplicate counts from four to seven crabs per treatment and time point.

Figure 2.1.6

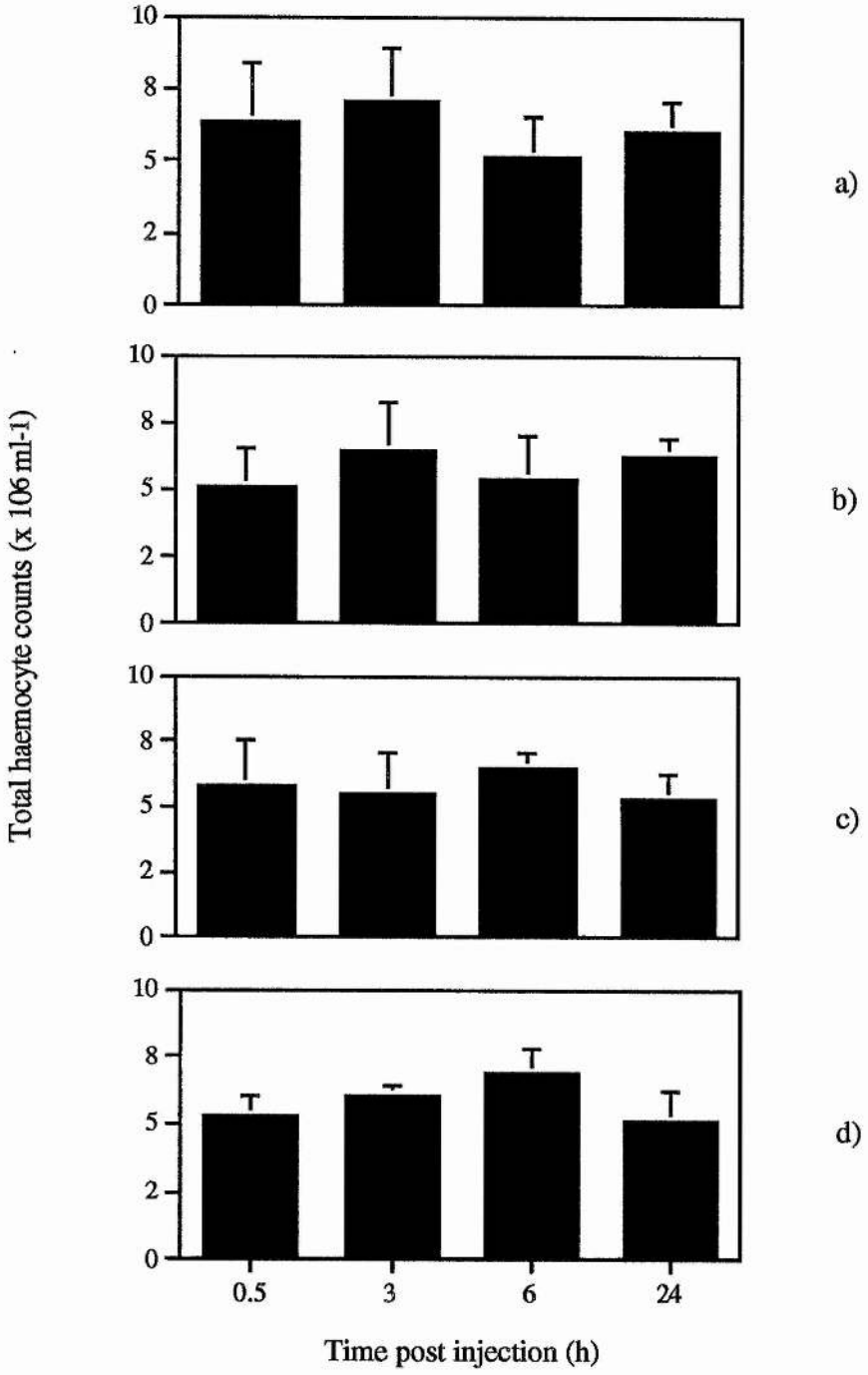
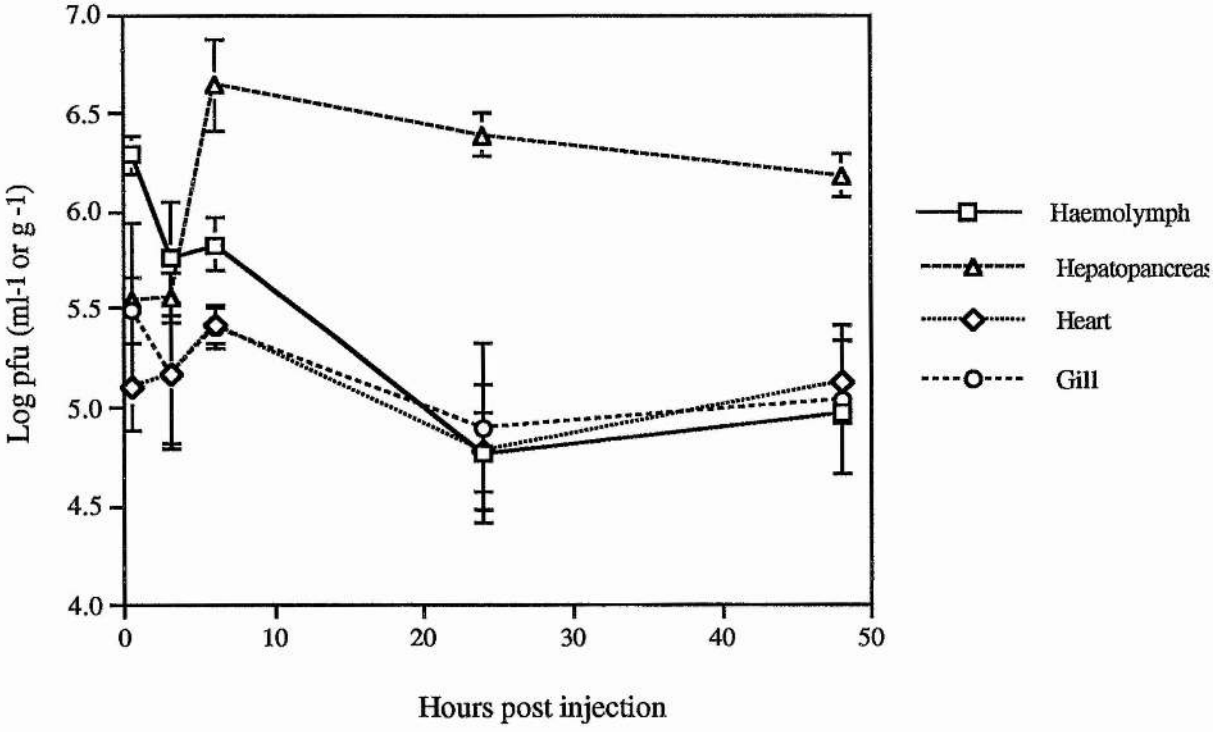

Total haemocyte counts (x 106 ml-1)
10
8
5
2
0
a)
b)
c)
d)
0.5
3
6
24
Time post injection (h)

Figure 2.1.7 Distribution of T_2 in the hepatopancreas, haemolymph, gills, or heart of *C. maenas* at different times after injection of ca. 2 x 10^8 pfu. Injections, bleeding, preparation of tissue samples and enumeration of phages were carried out as described in Materials and Methods. No further change in phage counts occured between 48 h and 72 h post injection. Values for 72 h post injection (not shown in graph) were: Hepatopancreas 6.08 $\pm$ 0.16 log pfu ml^{-1}, haemolymph 5.12 $\pm$ 0.37 log pfu ml^{-1}, gill 5.00 $\pm$ 0.302 log pfu ml^{-1} and heart 4.93$\pm$ 0.23 pfu ml^{-1}.

Figure 2.1.7


7.0
6.5
6.0
5.5
5.0
4.5
4.0
Log pfu (ml-1 or g-1)
0
10
20
30
40
50
Hours post injection
Haemolymph
Hepatopancreas
Heart
Gill

2.2 *IN VITRO* PROPHENOLOXIDASE ACTIVATION IN *C. MAENAS* HLS BY VIRUSES

Introduction

It has now been established that *C. maenas* can recognize and clear certain viruses *in vivo* (Chapter 2.1). However, it remains unclear whether or not clearance is mediated by humoral factors, or factors derived from the haemocytes. Clearance of dyes (Fontaine & Lightner, 1964; Mullainadhan *et al.*, 1984) or bacteria (Smith & Ratcliffe, 1980a; 1980b; White & Ratcliffe, 1982) from crustacean haemolymph is mediated by the granular haemocytes. This clearance response is likely to be linked to the prophenoloxidase (proPO) activating system, which in crustaceans is situated in the granular and semigranular haemocytes (Smith & Söderhäll, 1986a). In crustaceans, the cascade can be activated by bacterial lipopolysaccharides or fungal β 1,3-glucans (Unestam & Söderhäll, 1977; Smith & Söderhäll, 1983; Söderhäll & Häll, 1984). It is therefore not unreasonable to propose that components of viral coats or envelopes, such as glycoproteins, may similarly trigger the cascade.

Some evidence of the involvement of PO in antiviral defence has been gathered from insects (Miranpuri *et al.*, 1992; Ourth & Renis, 1993). Miranpuri *et al.* (1992) showed that PO activity in nymphs of the grasshopper *Melanoplus sanguinipes* increased 4 days after infection with *M. sanguinipes* entomopoxvirus. However, these authors did not investigate defence mechanisms associated with the proPO cascade, such as melanisation and encapsulation of infected tissues in *M. sanguinipes*. Ourth & Renis (1993) reported that vesicular stomatitis virus (VSV) and herpes simplex virus I (HSV I) are inactivated by the haemolymph of the tobacco budworm, *Heliothis virescens* in the presence of active phenoloxidase (PO). When PO is inhibited by the addition of phenylthiourea (PTU), the antiviral activity is lost (Ourth & Renis, 1993). As yet, the ability of viruses to activate the proPO cascade *in vitro* has not been studied. This chapter therefore aims to investigate whether or not viruses activate proPO in *C. maenas in vitro*.

The bacteriophages T_2, T_4, Φ-111 and NCIMB 386 and the *Chlorella* phage PBCV-1 were chosen as test agents. The PBCV-1 phage is a complex virus, with more than 50 structural proteins, including three viral glycoproteins (van Etten *et al.*, 1991). The glycans of PBCV-1 glycoproteins are displayed on the virus surface (van Etten *et al.*, 1991), where they could be targeted by the humoral and cell-mediated response in a multicellular host, making this phage a particularly suitable model for studies on the *in vitro* activation of phenoloxidase.

Materials and Methods

Animals and HLS preparation

Specimens of *C. maenas* were collected and maintained as described in Materials and Methods in Chapter 2.1 (page 64).

The method for the preparation of haemocyte lysate supernatants (HLS) was modified from Chisholm & Smith (1992), Smith & Söderhäll (1983) and Söderhäll *et al.* (1986). Haemolymph (ca. 2.5 ml crab $^{-1}$) was collected into an equal volume of ice-cold, sterile cacodylate (CAC) I buffer (0.01 M sodium cocodylate, 0.45 M NaCl, 0.1 M sodium citrate, pH 7.0) (Smith & Söderhäll, 1983). Haemolymph samples from at least seven crabs were pooled and centrifuged at 2000 *g* for 10 min at 4°C. The haemocytes were washed once in sterile, ice cold CAC II buffer (0.01 M sodium cacodylate, 0.45 M NaCl, pH 7.0) (Söderhäll *et al.*, 1986), suspended in ca. 3 ml of sterile, ice-cold CAC III buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 0.01 M $CaCl_2.6H_2O$, pH 7.5) (Söderhäll *et al.*, 1986) and homogenized for 5 min on ice with a pre-chilled, sterile glass-piston homogenizer. The homogenate was clarified by centrifugation at 48, 000 *g* for 20 min at 4°C. Haemocyte lysate supernatants were kept on ice for no longer than 30 min until use.

Viruses

The marine phage NCIMB 398 and its host, the bacterium NCIMB 386, were obtained from the National Collection of Marine and Industrial Bacteria (Aberdeen, Scotland). The bacteriophages T_2, T_4, Φ-111 and NCIMB 398 were grown and purified as described in Materials and Methods in Chapter 2.1 (page 65-68). Prior to use in PO activation assays, they were further purified by adding 0.75 mg ml^{-1} w/w of CsCl (Sigma) to each phage suspension, followed by centrifugation at 114, 000 *g* for 24 h. The phage bands, formed in the resulting isopycnic gradients, were collected by side-puncture with a sterile 21 gauge syringe needle. The T-phages were suspended in sterile salt magnesium (SM) buffer, while the marine phages were suspended in sterile marine SM (MSM) buffer, prepared as described in Materials and Methods in Chapter 2.1 (page 65). The suspensions were re-centrifuged at 114, 000 *g* for 2 h to pellet the particles, and the phages re-suspended in 1 ml SM buffer (T-phages) or MSM buffer (marine phages). They were titred against their respective host strains as described in Chapter 2.1 (page 67). The resulting titres were ca. 4×10^9 ml^{-1} for T_2, ca. 2×10^9 ml^{-1} for T_4 and ca. 8×10^{10} ml^{-1} for Φ-111. Phage NCIMB 398 was inactivated by the purification process but was retained to assess PO activation by components of the inactive phage particles.

A purified isolate of the *Chlorella* phage PBCV-1was gifted by Dr. D. E. Burbank (University of Nebraska, USA) and a culture of the host alga, NC-64A, was gifted by Dr. P. Mc Auley (University of St. Andrews). The titre of PBCV-1, as determined by Dr. Burbank, was 4×10^{10} ml^{-1}.

Protein assays

The protein content of HLS was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standards. The Coomassie blue reagent and BSA were supplied by Pierce (Illinois, USA).

Assay of phenoloxidase activity

Phenoloxidase activity was assayed using a modification of the procedure described by Söderhäll *et al.* (1986). This entailed adding 25 µl of HLS to 25 µl of each virus suspension. For a positive control, the virus was replaced with 25 µl of 0.1 % trypsin (from bovine pancreas, BDH, Poole, Dorset) in CAC III. For negative controls, the virus was replaced with 25 µl of CAC III or MSM. To investigate proPO activation by remaining components of the host bacteria, virus was replaced with 25 µl of a preparation from a non-infected culture of *E. coli* C-600, which had been subjected to the same purification regime as the phages. To investigate activation of proPO by components of the host alga of PBCV-1, the virus was replaced with 25 µl of an NC-64 A lysate, prepared as described for HLS, but with SM as homogenizing buffer. After 25 minutes at 18°C, 25 µl of a solution of L-dihydroxyphenylalanine (L-dopa) (Sigma) (3 mg ml^{-1} w/v) (Smith & Söderhäll, 1983) in DW was added. After a further 30 min at 18°C, 75 µl of CAC III was added and absorbance measured at 490 nm, against a blank comprising 25 µl each of MSM, trypsin, L-dopa and 75 µl of CAC III. One unit of PO activity was defined as an increase in absorbance at 490 nm of 0.001 min^{-1} ml^{-1} mg $protein^{-1}$.

To determine whether increased absorbance at 490 nm was due to direct oxidation of L-dopa by viral components, pro-PO activation assays were set up to include phenylthiourea (PTU), an inhibitor of phenoloxidase (Sugumaran *et al.*, 1988). Suspensions of PBCV-1 or NCIMB 398 were diluted 1/10 either in sterile SM

alone or in sterile SM containing a final concentration of 0.01% PTU. The viruses were incubated with HLS and PO activity was determined as above. For positive controls, HLS was incubated with 0.1% trypsin, prepared in sterile SM buffer, with or without 0.01% PTU.

To determine the concentration of viral particles required to activate pro-PO, HLS was incubated with two-fold serial dilutions of PBCV-1 in SM buffer and assayed for PO activity, as above, using trypsin (1 mg ml^{-1} w/v) as the positive control and SM buffer as the negative control, as before.

Staining for PO activity on PBCV-1 proteins immobilized on nitrocellulose membranes

Component proteins of PBCV-1, or molecular weight markers (BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa), all from Pharmacia, Uppsala, Sweden) were subjected to discontinuous sodium dodecyl sulphate acrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to the method of Laemmli (1970). Briefly, the separating gel (12% acrylamide) comprised 3.35 ml of DW, 2.5 ml of 1.5 M tris-HCl (pH 8.8), 100 µl of 10% SDS, 4.0 ml acrylamide and N,N-methylene bisacrylamide (Bis) stock solution (30% and 0.8%, respectively), 50 µl of 10% ammonium persulphate (APS) and 5 ml N,N,N',N'-tetramethylethylenediamine (TEMED). The stacking gel (4% acrylamide) comprised 6.1 ml of DW, 2.5 ml of 0.5 M tris-HCl (pH 6.8), 100 µl of 10% SDS, 1.3 ml of acrylamide - Bis stock solution, 50µl of 10% APS and 10 µl of TEMED. These amounts were sufficient for two minigels. The running buffer (pH 8.3) comprised 1.8 g of tris, 8.64 g of glycine and 0.6 g of SDS made up to 600 ml with DW. Molecular weight markers (in running buffer) or PBCV-1 suspensions were added to 4 volumes of sample buffer (4.0 ml DW, 1.0 ml 0.5 M tris-HCl (pH 6.8), 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml β 2-mercaptoethanol and 0.2 ml

0.05% bromophenol blue) and incubated at 100 °C for 3 min. Electrophoresis was carried out with a Mini Protean II unit (BioRad Laboratories, Watford, Herts.) at 200 V for ca. 45 min until the dye-front reached the bottom of the gels. Gels were run in pairs and one of the gels was stained in 0.5% Coomassie brilliant blue, 40% methanol, 10% glacial acetic acid overnight and de-stained in 10% glacial acetic acid. The other gel was used for Western blotting onto nitrocellulose.

For Western blotting, the gel was pre-equilibrated for 30 min in Towbin buffer (25 mM tris, 192 mM glycine, 20% methanol) (Towbin, 1971), together with blotting paper and a 0.45 µm nitrocellulose membrane (both from Millipore Waters, Watford), cut to the same size as the gel. Blotting was performed on a NovaBlot semi-dry blotting unit (Pharmacia-LKB) at 65 V for 2.5 h. After transfer, the nitrocellulose membrane was cut into strips. The transferred proteins were visualized by staining one strip overnight in a solution of 0.1% Winsor & Newton india ink in phosphate buffered saline (PBS) (Oxoid, Basingstoke, Hampshire), containing 0.3% Tween-20, as described by Hancock & Tsang (1983). Staining of nitrocellulose membranes for phenoloxidase activity was modified from Aspán & Söderhäll (1991). Nitrocellulose strips with immobilised phage proteins were washed for 2 h at 37°C in blocking buffer (0.05M tris, 0.45 M Nacl, 5 mM EDTA, 0.05% Triton-X 100, 0.25% gelatin, pH 8.0), then washed briefly with CAC III, sealed separately into polyethylene bags and incubated with freshly prepared HLS (0.5 ml per strip) for 4 h at 4°C. The strips were then removed from the polyethylene bags and incubated in petri dishes with ca. 10 ml of L-dopa in DW (3 mg ml^{-1} w/v) overnight at 18°C. To check that staining was not due to oxidation of L-dopa by viral proteins, one of the nitrocellulose strips was incubated with CAC III buffer in place of HLS, followed by incubation in L-dopa as above.

Statistical analysis

Differences in PO activity between HLS samples incubated in trypsin, buffer controls, viruses or host controls were compared by one way ANOVA. Detailed comparisions were made between NC-64A, SM and PBCV-1, between C-600, SM, T_2, T_4, Φ-111 and NCIMB 386 and between trypsin and CAC III. The effect of PTU on PO activity, following treatment of HLS with trypsin, PBCV-1 or NCIMB 386, was analysed by one-tailed paired t-test. The decrease of PO activity with two-fold serial dilution of PBCV-1 was analysed by linear regression of log PO activity *versus* PBCV-1 dilution. In all cases, significance was accepted when $P < 0.05$.

Results

Pro-phenoloxidase activation by viruses or bacteriophages

Table 2.2.1 shows the results from a typical proPO activation experiment using a range of bacteriophages or the PBCV-1 phage. Of all the viruses tested, only PBCV-1 produced a significant increase in PO activity from 5.5 ± 3.4 units for the SM control or 1.46 ± 0.61 units for NC-64A to 246.7 ± 23.63 units for PBCV-1 ($P < 0.001$) (Table 2.2.1). The algal host NC-64A did not significantly activate proPO ($P < 0.05$) (Table 2.2.1) The bacterial host control, C 600, and the bacteriophages significantly activated proPO compared to the SM control ($P < 0.01$) (Table 2.2.1). The highest proPO activation was seen with NCIMB 386 (188.1 ± 19.5 units) but none of the phages led to significantly increased PO activity compared to the C-600 control ($P > 0.05$) (Table 2.2.1), indicating that proPO activation by these phages is likely to have been due to residual endotoxin. Strong activation of proPO by trypsin was evident (1174.4 ± 37.5 units for trypsin or 0.6 ± 0.4 units for the CAC III control, $P<0.001$), showing that the proPO cascade in

the HLS was active, but had not been spontaneously activated by the experimental procedure. A separate assay showed that increase in absorbance at 490 nm was significantly reduced when HLS was incubated with PBCV-1 diluted ten-fold in sterile SM, containing 0.01% PTU, as compared to sterile SM without PTU (27.8 ± 3.5 units or 85.9 ± 4.7 units, respectively, $P < 0.02$). This shows that the increase in absorbance was not due to direct oxidation of L-dopa by components of PBCV-1.

Figure 2.2.1 shows the titration of PO activity against two-fold serial dilutions of PBCV-1, starting with an input concentration of 1×10^9 particles ml^{-1}. The trypsin control shows strong PO activity (1305.6 ± 32.3 units), whereas the SM buffer control does not show appreciable PO activity (15.4 ± 1.8 units), indicating that the proPO cascade in the HLS was stable and inactive prior to addition of the virus (Figure 2.2.1). The logarithm of PO activity significantly decreased with decreasing viral concentration ($P < 0.001$). The lowest viral concentration giving significant ($P > 0.05$) PO activity corresponded to ca. 5×10^8 particles ml^{-1} mg $protein^{-1}$ ml^{-1} (corresponding to a viral dilution of 1/64 in Figure 2.2.1).

Figure 2.2.2 shows the result of L-dopa staining of PBCV-1 proteins immobilized on nitrocellulose. SDS-PAGE profiles of molecular weight markers, and PBCV-1 proteins are shown in lanes A and B, respectively (Figure 2.2.2). Lane C shows a nitrocellulose blot of PBCV-1 proteins, stained with india ink, while lane D shows an L-dopa stain of PBCV-1 proteins after incubation in HLS (Figure 2.2.2). By comparision, lane E shows an L-dopa stain of PBCV-1 proteins after incubation with CAC III to detect oxidation by viral proteins (control) (Figure 2.2.2). From lane C it is evident that most viral proteins were transferred onto the membrane (Figure 2.2.2). The appearance of additional bands, not visible in lane B, is probably due to the India ink stain, the sensitivity of which is higher than of Coomassie blue (Hancock & Tsang, 1983). In lane D, it can be seen that several viral proteins have

stained with L-dopa in the presence of HLS (arrows). The major viral protein, a 54 kDa glycoprotein (van Etten *et al.*, 1991), stained positively. The upper part of the blot is stained indistinctly, hence it is not clear whether or not there is positive staining of the two remaining viral glycoproteins at 135 kDa and 75 kDa (Figure 2.2.2). However, several other viral proteins were stained, showing relatively indiscriminate binding of L-dopa to viral proteins (Figure 2.2.2, arrows). The control (lane E) does not show any stain, indicating that L-dopa was not oxidised by viral proteins in the absence of PO (Figure 2.2.2).

Discussion

Work presented in this chapter indicates that pro-PO in *C. maenas* haemocytes is probably activated by components of the algal virus PBCV-1. Pro-phenoloxidase was also activated in the presence of the bacteriophages T_2, T_4, Φ-111 or NCIMB 398 but activity was not significantly higher than that present in a control for residual bacterial endotoxin. The detection of increased activity did not result from oxidation of L-dopa by PBCV-1 proteins, as shown by PTU inhibition assays. However, with PBCV-1, proPO activation was only achieved with concentrations above ca. 5×10^8 viral particles mg protein $^{-1}$ ml^{-1}.

Several PBCV-1 proteins stained with L-dopa after incubation with HLS. including at least one of the three viral glycoproteins, Vp54, which is the major protein component of PBCV-1 (van Etten *et al.*, 1991). Staining did not result from direct oxidation of L-dopa by blotted PBCV-1 proteins. It is therefore likely that L-dopa staining of PBCV-1 proteins was the result of PO activation by viral components. However, because phenoloxidase is a "sticky" protein (Söderhäll & Smith, 1986) it may bind non-specifically to viral proteins, once it has been activated. The viral component responsible for proPO activation by PBCV-1 could

therefore not be determined by staining blots of total viral proteins. As a next step, it would be necessary to fractionate the viral proteins and test them individually for proPO activation. Surface proteins of viruses can be identified by radioiodinating the viruses and subjecting them to re-centrifugation on density gradients to separate intact and disrupted particles (Bartholomew *et al*, 1978). Only surface particles of intact viruses will thus be labelled (Bartholomew *et al*, 1978). The particles can subsequently be disrupted and the proteins be separated and assessed for PO activation. Attempts to propagate PBCV-1 on its host alga, the *Chlorella* strain NC-64A, remained unsuccessful and the available material was limited, so that further work on proPO activation could not be carried out in the present study.

In vertebrates, the antibody-independent inactivation of viruses by components of the alternative or classical (via C_q binding) complement pathways has been investigated by several workers (e.g. Cooper *et al.*, 1976; Bartholomew *et al.*, 1978; Grewal *et al.*, 1980; Hirsch *et al.*, 1981; Hirsch, 1982; Reading *et al.*, 1995) and its potential importance for the defence of non-immune hosts has been emphasized (Grewal *et al.*, 1980; Hirsch, 1982; Reading *et al.*, 1995). However, the nature of the viral antigens which lead to complement-activation remains mostly unclear (Cooper *et al.*, 1976; Grewal *et al.*, 1980), although it is known that oligosaccharides with high mannose contents in influenza virus haemagglutinin bind a guinea-pig serum lectin, leading to activation of the classical complement pathway (Reading *et al.*, 1995). In the case of PBCV-1, it is probable that viral glycoproteins are involved in PO activation, but the nature of the capsid glycans remains unknown (van Etten *et al.*, 1991). Because of the diversity of viral glycoproteins and their probable absence on some viruses, such as the fish pathogen infectious pancreatic necrosis virus (IPN) (Dobos *et al.*, 1979), which can also infect crustaceans (Halder & Ahne, 1988), it is unlikely that recognition of specific carbohydrates mediates non-specific defense against viruses in crustaceans in a manner analogous to defence against bacteria and fungi.

Because the PBCV-1 virus was not viable, it could not be investigated whether or not proPO activation was associated with virus neutralisation. The presence of neutralisation factors in the haemolymph or tissues of crustaceans may be a component of antiviral defence and this will be investigated in the following chapter.

Table 2.2.1 PO activation in *C. maenas* HLS by phages and controls

[1] Treatment		[2] PO activity (units)
PBCV-1 (1 x 10^9 ml^{-1})		246.7 ± 23.6 $^{a***; b***}$
PBCV-1 control: NC-64A		1.5 ± 0.6 ^{aNS}
T_2 (1 x 10^7 ml^{-1})		99.8 ± 51.1 $^{a*, cNS}$
T_4 (1 x 10^7 ml^{-1})		61.7 ± 13.4 $^{aNS, cNS}$
NCIMB 398		188.1 ± 19.5 $^{a***, cNS}$
Φ 111 (2 x 10^8 ml^{-1})		36.3 ± 21.0 $^{aNS, cNS}$
bacteriophage control: C-600		129.4 ± 45.7 ^{a**}
Trypsin (3 mg ml^{-1})	(+ control)	1174.4 ± 37.5 $^{a***; d***}$
CAC III	(- control)	0.6 ± 0.4 ^{aNS}
SM buffer	(- control)	5.4 ± 3.4

[1] Twenty five microlitres of HLS, prepared in CAC III buffer as described in Materials and Methods, was incubated with 25 µl of virus, buffer of host control. After 20 min at 18 °C, 50 µl of L-dopa were added and the samples incubated for a further 35 min. Subsequently, 75 µl of CAC III buffer was added and absorbance read at 490 nm. The protein content of HLS was ca. 1.97 mg ml^{-1}.

[2] One unit of PO activity is defined as an increase of 0.001 in the absorbance at 490 nm min^{-1} ml^{-1} mg $protein^{-1}$. Significance: [a] significantly higher than SM buffer, [b] significantly higher than NC-64A (PBCV-1 only), [c] significantly higher than C-600 control extract (phages only), [d] significantly higher than CAC III (trypsin only), ** $P < 0.01$, *** $P < 0.001$, NS not significant.

Figure 2.2.1 Titration of PO activity with concentration of PBCV-1. A virus suspension containing ca. 4×10^{10} particles ml^{-1} was subjected to two fold serial dilutions and 25 µl of this suspension was incubated with 25 µl of *C. maenas* HLS and assayed for PO activity as described in Materials and Methods. The protein content of HLS was ca. 1.35 mg ml^{-1}. Significant PO activation was detected at virus dilutions of $\leq$ 1/64, corresponding to at least 5×10^8 viruses mg $protein^{-1}$ ml^{-1}.

Figure 2.2.1

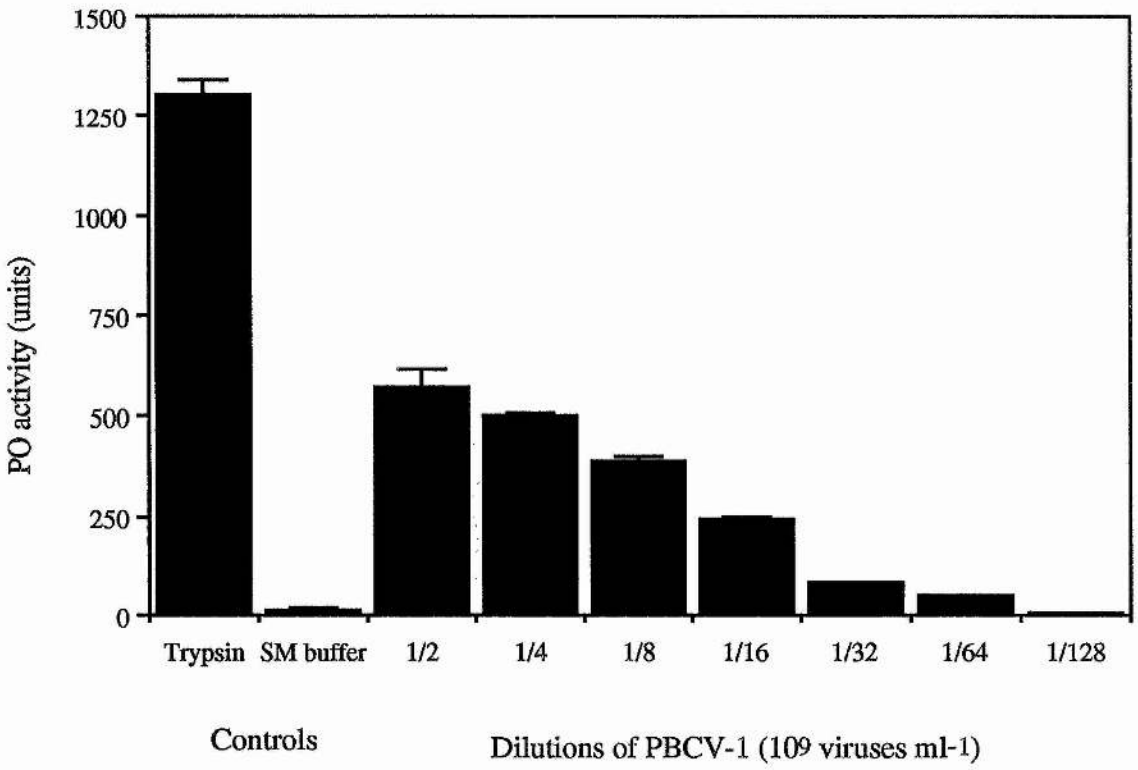

1500
1250
1000
750
500
250
0
PO activity (units)
Trypsin
SM buffer
1/2
1/4
1/8
1/16
1/32
1/64
1/128
Controls
Dilutions of PBCV-1 (109 viruses ml-1)

Figure 2.2.2 Staining of PBCV-1 proteins, immobilised on nitrocellulose. The viral proteins were subjected to SDS-PAGE as described in Materials and Methods, blotted onto nitrocellulose strips and stained for total protein or L-dopa oxidation as described in Materials and Methods. Arrows on the far right indicate bands in Lane D. The main viral protein (a 54 kDa glycoprotein) is designated Vp54.

Lane A: SDS-PAGE of molecular weight markers (BSA, 67 kDa; ovalbumin, 43 kDa and chymotrypsinogen, 25 kDa), Coomassie blue stain.

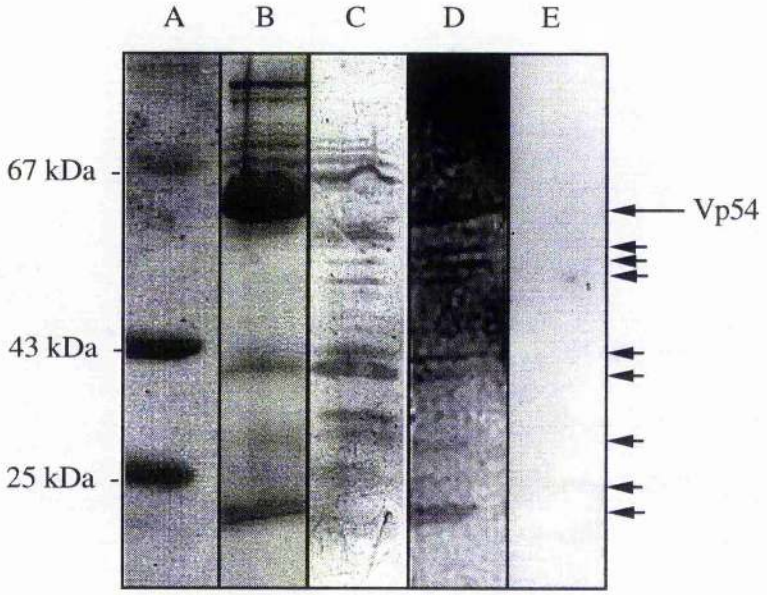
Lane B: SDS-PAGE of PBCV-1 proteins, Coomassie blue stain.

Lane C: India ink stain of PBCV-1 proteins blotted onto nitrocellulose.

Lane D: L-dopa stain of PBCV-1 proteins after incubation with HLS (arrows).

Lane E: Control blot: L-dopa stain of PBCV-1 proteins incubated with CAC III buffer in place of HLS.

Figure 2.2.2



2.3. *IN VITRO* NEUTRALISATION OF VIRUSES AND BACTERIOPHAGES BY HAEMOLYMPH COMPONENTS, DIGESTIVE JUICE OR TISSUE EXTRACTS OF THE SHORE CRAB, *CARCINUS MAENAS*.

Introduction

It has been shown that *C maenas* is capable of recognizing and partly eliminating certain particles from the circulation (Chapter 2.1) and that some viral particles may activate prophenoloxidase (proPO) (Chapter 2.2). However, it is not known whether or not antiviral factors are present in the haemocytes, plasma, tissues, or digestive juice of *C. maenas*.

Antiviral activity *in vitro* has been reported in the plasma or serum of the blue crab, *Callinectes sapidus* (McCumber *et al.*, 1979), the snail, *Biomphalaria glabrata* (Cheng *et al.*, 1984), the oyster *Crassostrea virginica* (Bachere *et al.*, 1990) and the tobacco bud worm *Heliothis virescens* (Ourth & Renis, 1993). An intrinsic antiviral factor is also present in the haemolymph of silk worm larvae, *Bombyx mori*, infected with an entomopoxvirus (Funakoshi & Aizawa, 1989).

Few workers have looked at antiviral activity in tissues or body fluids other than haemolymph. However, neutralising factors may be contained within haemocytes, tissues or gut juice where they may not come into direct contact with viruses present in the haemolymph, but prevent infection by viruses through the gut barrier or infestation of tissues (Tinsley & Harrap, 1978). Antiviral substances in the digestive juice or gut epithelial cells may be of special importance for host antiviral defence, because many invertebrate viruses infect their hosts via the food chain (Tinsley & Harrap, 1978). Uchida *et al.* (1984) have isolated an antiviral protein from the gut-juice of *B. mori*, infected with a nuclear polyhydrosis virus. A number of tissues of decapod crustaceans, including the hepatopancreas, heart and gill, are also known to contain fixed haemocytes (Johnson, 1978) which may act in antiviral defence. In the present chapter, the *in vitro* neutralising capacity of haemocyte extracts, plasma, digestive juice or extracts of the above tissues of *C. maenas* is tested against several bacteriophages and animal viruses.

The coliphages T_2 and T_4 were selected for this study, because these phages are rapidly cleared from the haemolymph of *C. maenas* (Chapter 2.1). Furthermore, these phages have been used for neutralisation studies in several invertebrates (McCumber *et al.*, 1979; Cheng *et al.*; 1983, Bachere *et al.*, 1990). Two marine bacteriophages from British waters, Φ-111 and NCIMB 398, which may be encountered by *C. maenas* in its natural environment, were also included as test agents. In addition, a number of animal viruses, which may be closer related to pathogens of *C. maenas,* were used. These include the fish pathogen infectious nuclear polyhydrosis virus (IPNV or IPN), *Autographa californica* multiple nuclear polyhydrosis virus (AcMNPV or NPV), herpes simplex virus I (HSV-1) and parainfluenza. IPN is known to infect a range of marine and freshwater invertebrates, including crustaceans (Hill, 1982; Bovo *et al.*, 1984; Halder & Ahne, 1988; Mortensen, 1993), which may act as vectors for transmission (Halder & Ahne, 1988; Mortensen, 1993). NPV is of potential use in insect pest control, which has lead to concern that it may infect non-target species, including crustaceans. (Couch & Martin, 1984). HSV I and parainfluenza have been reported to be neutralized by *H. virescens* plasma *in vitro* (Ourth & Renis, 1993).

Materials and Methods

Animals and preparation of haemolymph or tissue extracts

Specimens of *C. maenas* were collected and maintained as described in Materials and Methods in Chapter 2.1 (page 64).

Haemocyte lysate supernatants (HLS) were prepared according to the method of Chisholm & Smith (1992). Briefly, haemolymph samples from at least seven crabs (ca. 2.5 ml crab $^{-1}$) were collected in an equal volume of ice-cold, sterile marine anticoagulant (MA), pooled and centrifuged at 2000 *g* for 10 min at 4°C. The haemocytes were washed in

ice-cold, sterile CAC I buffer, suspended in 3 ml sterile *Carcinus* saline (CS) and homogenized for 5 min on ice with a pre-chilled, sterile glass-piston homogenizer. The homogenate was clarified by centrifugation at 48, 000 *g* for 20 min at 4°C.

For plasma samples, the crabs were bled as described in Chapter 2.1 (page 68) and the haemocytes were removed immediately after bleeding by centrifugation at 600 g for 10 min at 4°C. The plasma was transferred to fresh sterile pre-chilled tubes and kept on ice until use. Samples from at least three crabs were pooled prior to antiviral assays.

For preparation of digestive juice or tissue extracts, *C. maenas*, fed 48 h or 72 h previously, were chilled on crushed ice for ca. 10 min and killed as described in Chapter 2.1 (pages 69-70). Digestive juice was drawn through a blunted and bent 23 gauge ethanol-sterilized syringe needle, inserted into the pyloric stomach through the oral orifice. The digestive juice was stored in sterile tubs on ice, pooled with digestive juice collected from at least two other crabs, and clarified by centrifugation at 2000 *g* for 15 min at 4°C. Samples of hepatopancreas, gill, heart or gut were removed with sterile scalpels and forceps and suspended separately in 3 volumes of sterile CS at 4°C. For each tissue, samples from three crabs were pooled and homogenized for five minutes with sterile glass piston homogenizers at 4°C. The tissue extracts were clarified by centrifugation at 48, 000 *g* for 20 min at 4°C. All samples were used immediately or stored at -20°C for a maximum of two weeks before use in antiviral assays.

Protein and phenoloxidase assays

The protein content of the samples was determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standards. The Coomassie blue reagent and BSA were supplied by Pierce (Illinois, USA). Phenoloxidase of HLS samples was determined as described in Chapter 2.2 (page 95).

Viruses

The coliphages T_2, T_4 and the marine *Pseudomonas* phage Φ–111, were propagated and purified as described in Chapter 2.1 (page 64-67). The marine phage NCIMB 386 was similarly propagated. However, as this phage was inactivated by the purification method described in Chapter 2.1, cultures of this phage were instead clarified by centrifugation at 48, 000 *g* for 20 min at 4 °C. All phages were titred as described in Chapter 2.1 (page 67).

IPN virus and Bf (Bluegill fibroblast) cells were gifted by Drs. P.F. Dixon and K. Whey, MAFF Fish Diseases Laboratory, Weymouth. The host cell line was grown in sterile Glasgow Minimal Essential Medium (G-MEM) (Gibco, Paisley, Scotland), supplemented with 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 5% sterile fetal calf serum FCS (all from Gibco). The cells were grown as monolayers in 45 cm^2 tissue culture flasks (Corning, New York, USA), in sealed boxes in a 5% CO_2 atmosphere at 25°C. Host cell cultures were passaged twice weekly by washing the monolayers twice with sterile phosphate buffered saline (PBS) (Oxoid), covering each with 1 ml of sterile 10 mM EDTA and 0.02% trypsin (final concentration) in PBS, agitating the flasks for 30 seconds and sub-culturing 1 ml of the suspended cells into a new 45 cm^2 flask, containing ca. 4 ml of medium. When the monolayers had reached confluence, usually 24 h after passage, they were washed once with PBS and overlaid with a thin film (ca. 1 mm) of IPN in PBS (ca. 1 x 10^4 viruses ml^{-1}). After incubation for 1 h at 15 °C, to allow the virus to absorb to the cells, ca 5 ml of G-MEM, supplemented with 2% FCS, rather than 5% FCS, were added to the flasks and they were incubated for 4 days in sealed boxes in a 5% CO_2 atmosphere at 15°C until the monolayer showed signs of disruption. The supernatants were collected and clarified by centrifugation at 600 *g* for 10 min at 18°C. The virus was titred by the 50% tissue culture infective dose ($TCID_{50}$) assay as described below.

NPV virus and host cell line Sf 9 (*Spodoptera frugiperda*) were gifted by Prof.R. Hay, University of St. Andrews. The host cell line was grown in suspension

culture in ca. 100 ml of sterile TC 100 medium (Gibco), supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and 5% sterile FCS (all from Gibco). The cultures were incubated in spinner flasks (Gibco) at 27 °C and passaged twice weekly, by sub-culturing into ca. 4 volumes of fresh medium. The number of cells was determined with an Improved Neubauer haemocytometer, and freshly passaged cultures were infected with NPV in TC-100 supernatant at a multiciply of infection (moi) of ca. 0.01. The virus was grown in the same conditions as the host cell line and budding virus (non-occluded phenotype) was collected 1 week after infection from the supernatant after centrifugation at 600 g for 10 min at 18°C. The virus was titred by the $TCID_{50}$ assay as described below.

Parainfluenza virus, HSV I and Vero cells were gifted by Dr. R. Randall, University of St. Andrews. Vero cells were grown in monolayers in G-MEM, supplemented as above (5% FCS). The monolayers were incubated in a 5% CO_2 atmosphere in a CO_2 incubator and passaged twice weekly as described for Bf cells. The virus stock solutions were used directly for $TCID_{50}$ titration and antiviral assays as described below.

Titration of animal viruses

The 50% tissue culture infective dose ($TCID_{50}$) was determined as described in White & Fenner (1986). Briefly, viruses were serially diluted to 10^{-8} in the appropriate medium (TC-100 for NPV or G-MEM for IPN, HSV-1 or parainfluenza), containing 2% FCS for IPN or 5% FCS for HSV I, parainfluenza or NPV. One hundred microlitres of the first dilution were added to each well of the first column of a 96 well microtitre plate (Corning), as shown in Figure 2.3.1. One hundred microlitres of the second dilution was added to each well of the next column and the procedure was continued until all dilutions had been dispensed in this manner (Figure 2.3.1). The remaining columns of the microtitre plate received 100 µl of medium to act as cell viability controls (Figure 2.3.1). One hundred microlitres of a suspension of host cells in medium (ca. 1 x 10^5 cells ml^{-1}) were added to each well and the plates incubated as appropriate. A cytopathic effect (cpe) was detected

directly in the case of NPV, with the appearance of polyhedra within the host cells, and also with HSV I and parainfluenza. Plates with IPN virus were stained as follows: The medium was aspirated, 100 µl of neutral buffered formalin were added to each well, the cells were fixed for 1 h and the formalin was replaced with 100 µl of 0.1% crystal violet in PBS. The dye was removed after 30 min. Healthy monolayers stain dark violet whereas infected monolayers remain clear (Figure 2.3.1).

An example for determining $TCID_{50}$ by microtitre assay is also given in Figure 2.3.1. The proportionate distance (PD) of % infected wells is calculated as:

$$PD = \frac{(\% \text{ at dilution next above } 50\%) - (50\%)}{(\% \text{ at dilution next above } 50\%) - (\% \text{ at dilution next below } 50\%)}$$

and $TCID_{50}$ is determined as:

$$\log TCID_{50} = PD + \log \text{dilution next above } 50\%$$

Assay of antiviral activity

The bacteriophages T_2, T_4, Φ-111 or NCIMB 386 were diluted to a concentration of 2-3 x 10^4 pfu ml^{-1}. Ten microlitres of phage suspension were incubated with 90 µl of sample or control (CS) for 4 h at 18°C and enumerated by plaque assay as described in Chapter 2.1 (page 78 - 79). Antiviral activity is expressed as the percentage of pfu in the sample as compared to the control.

Activity of *C. maenas* extracts against animal viruses was analysed by neutralisation and titre reduction assay. For the neutralisation assay, successive two-fold dilutions of the sample in viral growth medium (VGM) were prepared in the first ten columns of a 96 well microtitre plate (50 µl per well). Fifty microlitres of the desired virus suspension in VGM

(ca 1 x 10^4 viruses ml^{-1}) were added to the samples in each well in the upper four rows of the plate. VGM was substituted for the virus suspension in the lower four rows. One hundred microlitres of the host cell suspension in VGM (ca 5 x 10^5 cells ml^{-1}) were added to each well. This arrangement divided the microtitre plate into quadruplicate assays as shown in Figure 2.3.2. This method quantifies the antiviral effect of the sample relative to the cytotoxic effect (White & Fenner, 1986). The relative antiviral activity is expressed as:

$$R = \frac{\text{mg ml}^{-1}\text{of sample protein cytotoxic for 50\% of cells}}{\text{mg ml}^{-1}\text{of sample protein giving 50\% antiviral protection}}$$

For the titre reduction assay, *C. maenas* extracts or CS controls were diluted ten-fold in VGM. Nine volumes of sample were added to one volume of stock virus to give final concentrations of ca. 1 x 10^7 ml^{-1} (IPN), ca. 3 x 10^7 ml^{-1} (NPV), ca. 1 x 10^6 ml^{-1} (parainfluenza) or ca. 1 x 10^4 ml^{-1} (HSV). The virus-sample suspensions were incubated for 4 h, at 15°C, in the case of IPN, or at 18°C, in case of NPV, parainfluenza or HSV. The viruses were subsequently titred as shown in Figure 2.3.1. The determination of $TCID_{50}$ was based on quadruplicate wells.

Analysis

Reduction in the percentage titres of bacteriophages were analysed by ANOVA (Campbell, 1982). A reduction of at least one log unit and an R value of 10 or above (White & Fenner, 1986) were taken as indicative of a positive antiviral response against animal viruses.

Results

Table 2.3.1 shows the percentages of titres of bacteriophages incubated in *C. maenas* extracts as compared to CS controls. All phages retained more than 89% of their activity

after incubation with *C. maenas* extracts (Table 2.3.1), hence *C. maenas* appears to display no neutralising activity against these phages *in vitro*.

Table 2.3.2 shows the effect of gut juice of *C. maenas* on NPV or parainfluenza. Complete inactivation (log reduction 7.5 from a log titre of 7.5) was observed for NPV, whereas a reduction of 2.7 log units was obtained for parainfluenza (Table 2.3.2). However, in a repeat experiment, neutralisation or reduction in titre of the parainfluenza virus by *C. maenas* gut juice was not evident and the R-value for NPV was only 2 (Legend to Table 2.3.3). For parainfluenza, the R value similarly was < 10, indicating that there is no specific antiviral activity, compared to cytotoxicity, against this virus in *C. maenas* gut juice (Table 2.3.2).

Table 2.3.3 shows the $TCID_{50}$ titres for viruses incubated with CS or *C. maenas* extracts. Total inactivation of NPV by the gut juice of *C. maenas* was observed, but the titres of IPN, NPV, Parainfluenza or HSV-1 were not reduced by HLS, plasma, or extracts of the hepatopancreas, heart, gill or midgut of *C. maenas*. Similarly, neither IPN nor HSV-1 were neutralized by *C. maenas* digestive juice and, IPN, NPV, Parainfluenza or HSV-1 were not neutralized by HLS, plasma or extracts of the hepatopancreas, heart, gill or midgut of *C. maenas*. The HLS used for the HSV-1 neutralisation assay had a phenoloxidase activity of 7447.9 units $\pm$ 126.6 units (mean $\pm$ SE).

Discussion

In this study, plasma or extracts of the haemocytes, hepatopancreas, heart, gut or gills of *C. maenas* were found to have little or no antiviral activity against the bacteriophages T_2, T_4, Φ-111 or NCIMB 398. Likewise, activity was not detected against the animal viruses IPN, NPV, parainfluenza or HSV-1. However, the digestive juice of *C. maenas* fully inactivated NPV, and partly inactivated parainfluenza, but the data were not consistent for repeat experiments. In one experiment with NPV, and in both experiments with

parainfluenza, R values were found to be < 10, indicating absence of specific antiviral activity (White & Fenner, 1986). Nonwithstanding, the non-specific inactivation of certain enveloped viruses by gut juice may have an important function in the antiviral defence of arthropods, as it may prevent infection by pathogenic viruses transmitted through the food chain (Tinsley & Harrap, 1978). This is the first study of antiviral activity in the gut juice of a crustacean, although previously an antiviral factor has been isolated from the digestive juice of the silkworm, *Bombyx mori*, infected with a nuclear polyhedrosis virus (BmNPV) (Uchida *et al.*, 1984). The Baculoviridae, which include BmNPV and AcNPV, also include many important pathogens of crustaceans, such as *Baculovirus penaei* (PdNPV) and Monodon baculovirus (PmNPV) (Johnson & Lightner, 1988). Antiviral factors in crustacean digestive juice against these viruses would therefore provide an important protection for these animals.

Antiviral factors have been described from the haemolymph of other arthropods (McCumber & Clem, 1979; Ourth & Renis, 1993). In particular, McCumber & Clem (1979) reported an 80 kDa "neutralising factor" against the coliphage T_2 in the haemolymph of the blue crab, *Callinectes sapidus*. Because *C. sapidus* is closely related to *C. maenas*, equivalent activity may have been expected to be present in the plasma of *C. maenas*. However, it has been noted in Chapter 2.1 that the two species differ in their ability to clear injected T_2 from the haemolymph (page 74). This difference in clearance ability may be due to subtle differences in the haemolymph composition of the two species.

In insects, neutralising activity against HSV-1, HSV-2, parainfluenza 3, cocksackie virus B2, vesicular stomatitis virus (VSV) or Sindbis virus (the latter two are insect-transmitted arboviruses) was detected in the haemolymph of the tobacco budworm (*Heliothis virescens*) by Ourth & Renis (1993). These authors found that antiviral activity against HSV-1 and VSV was dependent on active phenoloxidase (PO) and could be inhibited by collecting the haemolymph in the presence of phenylthiourea (Ourth & Renis, 1993). They detected HSV-1 inactivation with purified *H. virescens* PO and commercially available

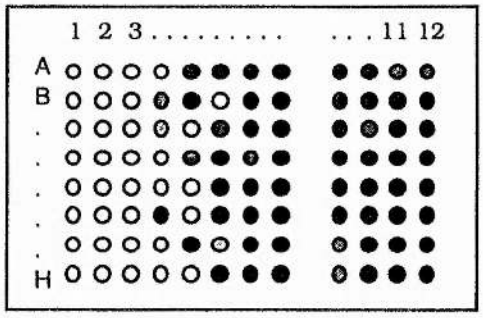
mushroom tyrosinase, but only in the presence of substrate (Ourth & Renis, 1993). Melanin formation is known to generate superoxide ions and H_2O_2 as by-products (Söderhäll, 1992), and the possibility that HSV-1 and VSV in the study by Ourth & Renis (1993) were killed by free oxygen radicals, rather than PO itself, cannot be ruled out. In *C. maenas*, proPO is present in haemocyte lysate supernatants (Smith & Söderhäll, 1983). However, in the present study, neutralisation of HSV-1 was not detected with HLS samples. It is possible that the proPO remained inactive in the presence of the virus, or that suitable substrates for PO activity were not present in the suspension. The ratio of virus titer to sample components (not indicated by Ourth & Renis, 1993) may be crucial. However, in the present work neutralisation assays with low concentrations of viruses (ca. 2×10^3 to 2×10^4 particles ml^{-1}) were carried out as well as titer reducton assays, using 100 to 1000 fold higher concentrations of viruses.

Absence of antiviral activity in the haemolymph has also been reported by Berheimer *et al.* (1952) for T_2 in the moth *Samia cecropia*, by Feng (1966) for *Staphylococcus aureus* phage 80 in the oyster, *Crassostrea viginica*, by Andersons (1993) for NPV in the moth, *Trichoplysia ni*, and by Ourth *et al.* (1994) for HSV-1 and VSV for inducible antibacterial proteins in *H. virescens* larvae. However, in some invertebrate species, antiviral substances have been described. For example, didemnins, from the Carribean tunicate *Tridemnum* spp., are active against Cocksackie virus, equine rhinovirus, HSV-1 and HSV-2 (Rinehart *et al.*, 1981) and halocyamine A, from haemocytes of the solitary ascidian *Halocynthia roretzi*, have antiviral properties against IPN (Azumi *et al.*, 1990). Tachyplesins, from the horseshoe crabs *Tachypleus tridentata* and *Limulus polyphemus*, have been found to be active against HIV (Morimoto *et al.*, 1991), VSV and Influenza A (Murakami *et al.*, 1991). However, these factors either require extraction from large amounts of material (1.4 g wet weight of *Tridemnum* tissue per µg of didemnin A, Rinehart *et al.*; 1978), or need to be applied in relatively concentrated form (125 µg ml^{-1} of tachyplesin for antiviral assays against VSV or Influenza A; Murakami *et al.*, 1991). Haemocyte lysate supernatants of *C. maenas* are known to contain several antibacterial proteins, and possibly peptides

(Chisholm, 1993). The use of these proteins or peptides in concentrated form in antiviral assays may indicate an *in vitro* antiviral response in *C. maenas*. In the next section of this thesis (Section 3), it will therefore be attempted to characterize and purify these antibacterial proteins or peptides.

Figure 2.3.1 Example for the calculation of the 50% tissue culture infective dose ($TCID_{50}$), using the microtitre plate method. Monolayers are grown and infected as described in Materials and Methods. Monolayers in rows A-H of the first 8 columns receive ten-fold serial dilutions of virus as follows: The cells in column 1 receive a 10^{-1} dilution of virus, the cells in column 2 receive a 10^{-2} dilution and so on up to the cells in column 8 which receive a 10^{-8} dilution. The monolayers in columns 10-12 receive sterile dilution medium to act as controls. After 4 days the monolayers are fixed and stained as described in Materials and Methods. The microtitre plate, stained with crystal violet, is shown in the upper part of the figure. Clear wells indicate monolayers which show a cytopathic effect (cpe), dark wells indicate healthy monolayers. The table and text below give the example for $TCID_{50}$ calculation from the wells shown. Clear wells are scored as positive, dark wells as negative. The cumulative scores of positive wells is counted from column 1-8, the score of negative wells from column 8-1. From the cumulative scores, the percentage of positive wells is determined and the $TCID_{50}$ calculated from this as indicated in the equations below the table.

Figure 2.3.1



dilution	pos. wells	neg. wells	Σ pos.	Σ neg.	pos./total	% pos.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
10^{-3}	8	0	16	0	16/16	100
10^{-4}	6	2	8	2	8/10	80
10^{-5}	2	6	2	8	2/10	20
10^{-6}	0	8	0	16	0/16	0

The proportionate distance (PD) is calculated from:

$$PD = \frac{(\%\ \text{positive at dilution.} > 50\%) - (50\%)}{(\%\ \text{positive at dilution} \geq 50\%) - (\%\ \text{positive at dilution} \leq 50\%)}$$

i.e. from the example above: $PD = \frac{80 - 50}{80 - 20} = 0.5$

$$\log TCID_{50} = PD + \log \text{dilution next above 50\% positive}$$

i.e. from the example above: $\log TCID_{50} = 0.5+(-4) = -4.5$

and $TCID_{50} = 10^{-4.5}$

The $TCID_{50}$ value is then converted to particles per ml, i.e. from this example $TCID_{50} = 3.16 \times 10^5$ pfu ml^{-1}.

Figure 2.3.2. Experimental setup for virus neutralisation and cytotoxicity assay. Figure 2.3.2a shows the division of the microtiter plate into sub-sections and Figure 2.3.2b shows the results of a hypothetical assay. The results are interpreted as follows:

Rows A-D:
Columns 1-10: Virus neutralisation assay. Healthy monolayers indicate neutralisation of virus by the sample,

Columns 11-12: Virus infectivity assay, control to ensure virus causes CPE at the dilution used.

Rows E-H:
Columns 1-10: Cytotoxicity assay. Indicates endpoint of cytotoxic effect (cte) of sample

Columns 11-12: Cell viability control. Healthy monolayers indicate that cells are viable and not cross-contaminated by virus.

Figure 2.3.2

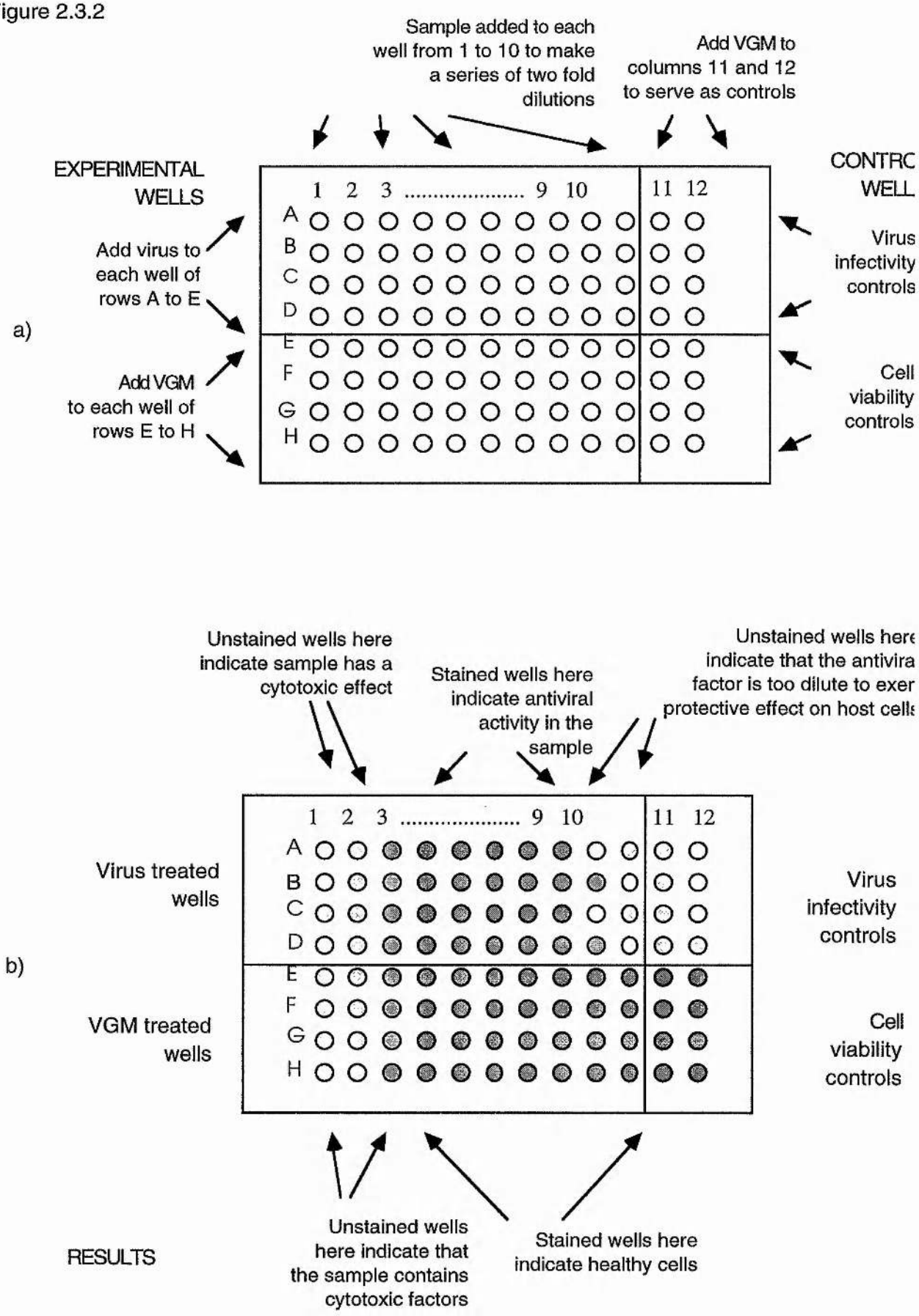

Sample added to each well from 1 to 10 to make a series of two fold dilutions
Add VGM to columns 11 and 12 to serve as controls
EXPERIMENTAL WELLS
CONTRC WELL
1 2 3 ..... 9 10
11 12
A
B
C
D
E
F
G
H
Add virus to each well of rows A to E
Add VGM to each well of rows E to H
Virus infectivity controls
Cell viability controls
a)
Unstained wells here indicate sample has a cytotoxic effect
Stained wells here indicate antiviral activity in the sample
Unstained wells here indicate that the antivira factor is too dilute to exer protective effect on host cells
Virus treated wells
VGM treated wells
Virus infectivity controls
Cell viability controls
b)
RESULTS
Unstained wells here indicate that the sample contains cytotoxic factors
Stained wells here indicate healthy cells

Table 2.3.1 Percentage titres of bacteriophages incubated with extracts from *C. maenas* as compared to CS.

[1] Sample	[2] Protein (mg ml^{-1})	[3] Bacteriophage T_2	T_4	Φ 1-1-1	NCIMB 386
CS	N/A	100	100	100	100
HLS	1.0	97.5 ± 0.2	92.3 ± 0.2	98.5 ± 0.1	104.5 ± 0.1
Plasma	34.0	97.5 ± 0.0	96.2 ± 0.0	113.3 ± 0.0	122.0 ± 0.1
Gutjuice	9.1	110.3 ± 0.2	97.4 ± 2.2	89.0 ± 0.0	89.4 ± 0.1
Hepatopancreas	10.9	100.0 ± 4.9	98.2 ± 2.2	100.2 ± 0.1	93.8 ± 0.0
Gill	3.7	94.1 ± 3.3	98.8 ± 1.4	95.8 ± 0.1	121.2 ± 0.1
Heart	4.3	95.7 ± 2.3	99.8 ± 4.3	93.0 ± 0.0	98.3 ± 0.1
Gut	1.4	94.1 ± 3.9	98.7 ± 6.5	110.8 ± 0.1	101.1 ± 0.1

[1] Phages were incubated in 9 volumes of sample and titred as described in Materials and Methods. The percentage titres of the phages was not significantly lowered by incubation in CS as compared to SM (T-phages) or marine SM (marine phages).

[2] Protein content of representative samples. N/A = not applicable.

[3] Values represent mean percentages of the control mean titre ± SE. n = 5 for marine phages, n = 3 for T-phages.

Table 2.3.2 *In vitro* neutralisation of NPV or parainfluenza by gut juice of *C. maenas*.

	NPV	Parainfluenza
[1] Protein (mg ml^{-1})	9.1	9.1
[2] log $TCID_{50}$	7.5	6.0
[3] log reduction	7.5	2.8
[4] R	27.0	4.0

1 Protein concentration of undiluted gut juice.

2 Titre of stock virus. The $TCID_{50}$ values are based on quadruplicate wells.

3 Reduction in the titre of 100 µl stock virus incubated in nine volumes of gut juice, diluted ten-fold in viral growth medium, for 4 h at 18°C. Final concentration of viruses in the sample mixtures was 3.2×10^6 for NPV or 1×10^5 for parainfluenza and final concentration of gut juice protein in the sample mixtures was 0.8 mg ml^{-1}. Viruses were titrated as above.

4 The R-values were detemined as described in Material and Methods. The 50 % cytotoxic concentration of protein was 1.01 mg ml^{-1} in case of the Sf 9 cells or > 0.45 mg ml^{-1} in case of the Vero cells.

Table 2.3.3 $TCID_{50}$ values for viruses incubated with CS or *C. maenas* extracts.

[2] Suspension	[3] Sample protein (mg ml^{-1})	[1] $TCID_{50}$ IPN	NPV	Parainfluenza	HSV1
VGM	N/A	$10^{6.6}$	$10^{7.5}$	$10^{6.0}$	$10^{4.0}$
CS	N/A	$10^{6.8}$	$10^{7.4}$	$10^{6.0}$	$10^{3.7}$
HLS	1.7	[4]$10^{7.0}$	$10^{7.7}$	[4]$10^{5.7}$	[4;5] N
Plasma	33.9	[4]$10^{6.5}$	[4;5] N	[4;5] N	[4;5] N
Gut juice	8.9	[4]$10^{6.8}$	[4;6] 0	[4;6]$10^{5.7}$	[4;5] N
HP	18.8	[4]$10^{7.0}$	[4]$10^{7.6}$	[4]$10^{6.0}$	[4;5] N
Gills	5.1	[4]$10^{6.4}$	$10^{7.3}$	$10^{6.0}$	$10^{4.0}$
Heart	3.4	[4]$10^{6.8}$	$10^{7.4}$	$10^{5.7}$	$10^{4.0}$
Gut extract	1.1	[4]$10^{6.7}$	$10^{7.7}$	$10^{5.7}$	$10^{3.7}$

[1] Values are based on quadruplicate wells.

[2] Viruses were initially titrated in viral growth medium (VGM). *Carcinus* saline (CS) or extracts of *C. maenas* were ten-fold diluted in VGM.

[3] Representative values. N/A = not applicable.

[4] Neutralisation assay negative.

[5] N: titre not determined.

[6] With this sample (protein ca. 8.9 mg ml^{-1}), no reduction in titre of parainfluenza was observed and parainfluenza neutralisation assay was negative. For NPV, R was 2.0.

3. ANTIBACTERIAL FACTORS IN THE HAEMOCYTES OF THE SHORE CRAB, *CARCINUS MAENAS*

3.1 OPTIMISATION OF ANTIBACTERIAL ASSAYS

Introduction

As a prerequisite for the purification of antibacterial factors from *C. maenas* HLS, it was necessary to optimise the sensitivity of the antibacterial assays and to investigate alternative assay methods which require lower sample volumes. Previous investigations of *in vitro* antibacterial activity in *C. maenas* HLS used a spread-plate neutralisation assay as described by Chisholm & Smith (1992). The aim of the present investigation was to increase the sensitivity of this assay and to reduce the amount of sample required for antibacterial assays by adapting a radial diffusion assay (Lehrer *et al.*, 1991) for use with *C. maenas* HLS.

The marine Gram negative bacterium, *P. immobilis*, was chosen as a sentinel organism because it is known to be susceptible to antibacterial proteins in *C. maenas* HLS (Chisholm & Smith, 1992; Chisholm, 1993). The *E. coli* strains D21, D22 and D31 were chosen because they are frequently used in investigations of antibacterial factors in insects (Hultmark *et al.*, 1980; Boman, 1994). None of the bacterial strains included in these investigations are pathogenic to humans.

Materials and Methods

Animals and HLS preparation

Specimens of *C. maenas* were collected and maintained as described in Chapter 2.1 (page 64). Haemocyte lysate supernatants were prepared in sterile tris-buffered 3.2% NaCl (0.05 M tris, pH 7.2) and assayed for protein as described in Chapter 2.2 (page 95). Phenylthiourea (PTU) was added to the homogenising buffer to a final concentration of 0.01% (Chisholm, 1993).

Following the optimisation of the assay procedures, a sample of HLS was divided into three aliquots. Two aliquots were used for the spread plate assays, with or without lysozyme, and the remaining aliquot was used for the radial diffusion assay to allow a direct comparision between these methods.

Bacteria

P. immobilis (NCIMB 308) was maintained at 4°C on slopes of marine agar (Difco). For antibacterial assays the bacteria were grown to log-phase for 16-18 h at 18°C in marine broth (Difco). They were harvested from the broth by centrifugation at 1900 *g* for 10 min at 18°C, washed once in sterile 3.2% NaCl and resuspended in sterile 3.2% NaCl to an absorbance of ca. 0.5 at 570 nm, giving an approximate bacterial concentration of ca. $1x10^8$ ml^{-1}.

The *E. coli* K12 derived strains D21 (CGSC 5158), D22 (CGSC 5163) and D31 (CGSC 5165) were maintained on slopes of Luria Bertani (LB) agar (Fluka) at 4°C. For use in antibacterial assays, they were grown in LB broth (Fluka) for ca 16 h at 37°C. To ensure log-phase growth, an equal volume of fresh broth was then added and the cultures were grown for a further 4 h at 37°C. Bacteria were harvested as described above, washed and re-suspended in phosphate buffered saline (PBS) (Oxoid), prior to adjustment of absorbance at 570 nm. An absorbance reading of 0.5 gives an approximate bacterial concentration of $1x10^8$ ml^{-1}.

The spread plate assay

The spread plate assay was modified from Chisholm & Smith (1992). Briefly, 90 µl of HLS were incubated with 10 µl of *P. immobilis* to give a final concentration of ca. 1 x 10^4 colony forming units (cfu) ml^{-1}. In controls, HLS was

replaced with 90 µl of sterile *Carcinus* saline (CS). After 4 h incubation at 18°C, 900 µl of sterile 3.2 % NaCl were added to each preparation and 100 µl aliquots plated onto marine agar (Difco) plates. Plates were incubated for 72 h at 25 °C. Antibacterial activity was expressed as the Survival Index (SI) (Wardlaw & Unkles, 1978):

$$\frac{\text{Number of cfu at time } t_1}{\text{Number of cfu at time } t_0 \text{ x100}}$$

Assays were carried out in triplicate and results are expressed as mean ± SE.

To increase the sensitivity of the assay, the volume of HLS or buffer control was reduced to 80 µl and 10 µl of a solution of chicken egg-white lysozyme (Sigma L-6876; Chalk *et al.*, 1994) was added to each to give a final concentration of 1 mg lysozyme ml^{-1}. An additional control was set up with 90 µl of buffer alone. Otherwise, bacterial concentrations and assay conditions were as above.

The radial diffusion assay

The radial diffusion assay was modified from Lehrer *et al.* (1991). An underlay of 1% (final concentration) low-electroendoosmosis type agarose (Sigma) and 0.02% (final concentration) Tween-20 (Sigma) was prepared in 10 ml aliquots, sterilized by autoclaving and kept fluid in a waterbath at 45°C. Just prior to use, each aliquot was seeded with *P. immobilis* to a final concentration of ca. 2 x 10^6 bacteria ml^{-1} and poured into sterile square petri dishes (10 x 10 cm) to give a thickness of ca. 1.2 mm. The composition of the underlay buffer consisted of either marine broth or one of the following: 3.2% NaCl, CS or NaCl-free phosphate buffer (PB, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, pH 7.2), with or without marine broth at 10% final concentration. Wells of ca. 3 mm diameter were punched into the underlay and filled with 2.5 µl of HLS or sterile CS. The plates were incubated for

3 h at 18°C, prior to addition of an overlay consisting of an equal volume of double strength marine broth in 1% agarose. The plates were then incubated at 25°C for 24-48 h until clear zones were visible around the sample wells. Zones were measured with a ruler under a binocular microscope (Zeiss, Dresden, Germany), fitted with a graticule aligned to the ruler. Activity was expressed as area of the clear zone minus area of the well. Assays were carried out in triplicate and results were expresssed as mean $\pm$ SE.

To determine the effect of different bacterial concentrations on the sensitivity of the assay, an underlay consisting of 1% agarose in PB with 0.02% Tween-20 (final concentrations) was seeded with ca. 2 x 10^5 or 2 x 10^6 *P. immobilis*. Aliquots of 7.5 ml, rather than 10 ml, were used to pour round petri dishes. The diameter of the wells was decreased to ca. 2 mm and 2 µl of buffer control or HLS were added to each well. The plates were incubated and assessed for antibacterial activity as above. In a separate experiment, the underlays were seeded with *E. coli* D21, D22 or D31 to give final concentrations of ca. 2 x 10^4, 2 x 10^5 or 2 x 10^6 bacteria ml^{-1}. Samples were added as above, and after 3 h incubation at 18°C, an overlay consisting of 1% agarose in double-strength LB broth was added. Clear zones were noted after 16-18 h incubation at 37°C and antibacterial activity determined as described above.

Statistical analysis

Paired t-tests (Campbell, 1990) were used to analyze the effect of lysozyme on the SI of *P. immobilis*, incubated with CS or two-fold serial dilutions of HLS. Single factor ANOVA (Campbell, 1990) was used to analyse the effect of underlay composition on the sensitivity of the radial diffusion assay. For this test, values compared represented total areas, including well areas, to avoid inhibition zones of zero and resulting lack of sample variation where no clear zones were present around the wells. Independent ANOVA comparisions between different underlay

compositions were also carried out. Two-factor ANOVA was used to compare the inhibition zones of *E. coli* D21, D22 or D31 at different bacterial concentrations. For all statistical tests a probability level of ≤ 0.05 was accepted as significant.

Results

Spread plate assay

Addition of lysozyme to the spread plate assay increased the sensitivity of *P. immobilis* to antibacterial factors in *C. maenas* HLS (Figure 3.1.1). Buffer controls or dilutions of HLS incubated with or without lysozyme showed significant differences for sample dilutions of 1/32 ($p < 0.02$) and 1/64 ($p < 0.05$) (Figure 3.1.1). The SI of the controls remained unaffected ($p < 0.40$). Significant differences could not be determined where the survival indices for one of the treatments were zero, due to the lack of sample variation. In the presence of lysozyme, antibacterial activity was detectable at a total protein concentration of HLS of ca. 63 $\mu g\ ml^{-1}$ (SI 32.8 ± 4.0), whereas it was detectable at a concentration of ca. 126 $\mu g\ ml^{-1}$ in the absence of lysozyme (SI 79.1 ± 8.3) (Figure 3.1.1). These values repesent 1/64 and 1/32 dilutions of the sample, respectively (Figure 3.1.1). However, SI values comparable to a dilution of 1/64 of HLS in the presence of lysozyme (SI 32.8 ± 3.9) were reached at a dilution of 1/16 in the absence of lysoyme (SI 30.6 ± 3.9) (Figure 3.1.1). This indicates that the inclusion of lysozyme increased the overall sensitivity about 4-fold.

Radial diffusion assay

Table 3.1.1. shows the effect of underlay composition on the sensitivity of the radial diffusion assay against *P. immobilis*. There was a significant overall

difference between samples ($p<0.001$). Clear zones were not evident when the underlay contained normal strength marine broth or CS (Table 3.1.1). Large clear zones were evident in underlays prepared with marine broth ten-fold diluted in PB (Table 3.1.1), but these were significantly smaller than those in underlays prepared with PB alone ($p < 0.01$). Clear zones in underlays containing 3.2% NaCl (8.0 ± 0.4 mm^2) or marine broth ten-fold diluted in CS were also significantly smaller than those prepared with PB ($p < 0.01$). Accordingly, an underlay composition of 1% agarose in PB with 0.02% Tween-20 (final concentrations) was chosen for use in radial diffusion assays.

Figure 3.1.2. shows the titration of whole HLS against *P. immobilis* using the radial diffusion assay. Clear zones were detectable down to a total protein concentration of 126 μg protein ml^{-1}, which corresponds to a 1/32 dilution of the sample This indicates that the sensitivity of the radial diffusion assay is comparable to that of the spread plate assay in the absence of lysozyme, shown in Figure 3.1.1.

Experiments set up to determine the effect of bacterial concentration on inhibition zone size showed that, for *P. immobilis*, final concentrations of ca. 2×10^5 bacteria ml^{-1} in the underlay resulted in slow growth and diffuse lawns. Figure 3.1.3. shows titres of two-fold serial dilutions of whole HLS against *E. coli* D31 at concentrations of ca. 2×10^6, 2×10^5 or 2×10^4 bacteria ml^{-1}. Activity was not discernible at dilutions greater than 1/2, for bacterial concentrations of ca. 2×10^6 ml^{-1}, whereas at a concentration of ca. 2×10^4 bacteria ml^{-1}, activity was still present at a dilution of 1/8 (Figure 3.1.3). Table 3.1.2 shows that the inhibition zones for whole HLS increased with decreasing bacterial concentration for all three *E. coli* strains tested ($p<0.001$). Table 3.1.2 also shows that *E. coli* D22 is the most sensitive of the three strains to antibacterial factors in *C. maenas* HLS (difference between strains $p < 0.001$).

Discussion

Two sensitive methods have been adapted for assaying antibacterial activity in *C. maenas* HLS. Firstly, the addition of lysozyme to suspensions of *P. immobilis* in *C. maenas* HLS, used for spread plate assays, resulted in an about four-fold increase in sensitivity, compared to assays using *P. immobilis* incubated in *C. maenas* HLS alone. A synergistic effect of lysozyme with factors acting on the outer membrane of Gram negative bacteria has previously been reported for *Hyalophora cecropia* attacins by Engström *et al.* (1984). Chalk *et al.* (1994) included lysozyme in a modification of the inhibition zone assay of Hoffmann *et al.* (1981) for the purification of defensins from the mosquito *Aedes aegypti.* One disadvantage of incorporating lysozyme in antibacterial assays is that it precludes the use of Gram positive bacteria, which are not protected by an outer membrane from lysozyme mediated cleavage of peptidoglycans.

The spread plate assay requires relatively large volumes of reagents and sample. By contrast, radial diffusion assays require smaller sample volumes (2 µl per well), and are thus useful for assaying HLS fractions obtained during protein purification. The assay, devised by Hoffmann *et al.* (1981), entails adding sample to wells punched into thin plates of nutrient agar pre-seeded with the test bacterium. Clear zones are measured after incubation. Previous attempts to adapt such an assay for use with *C. maenas* HLS have been unsuccessful due to melanisation in the sample wells (Chisholm, 1993). In the present study, melanisation was prevented by the addition of PTU, a known inhibitor of phenoloxidase (Sugumaran *et al.*, 1988), to the homogenizing buffer. However, clear zones did not appear around sample wells in lawns of *P. immobilis* which were prepared with marine agar. Addition of lysozyme to the lawns at final concentrations of 1 mg ml^{-1} or 5 mg ml^{-1} also failed to give inhibition zones. The main problem with this assay appears to be the

interaction of the sample with the agar (Lehrer *et al.*, 1991; Boman, 1994). To minimize interactions with charged components of agaropectin, Lehrer *et al.* (1991) recommend replacing the agar with low-electroendoosmosis type agarose. Accordingly, a two-layer radial diffusion assay, based on the procedure of Lehrer *et al.*, (1991), was adapted for use with *C. maenas* antibacterial proteins. However, clear zones were not obtained with *C. maenas* HLS when agarose underlays were prepared with normal-strength marine broth or high salt buffers. It is known that high ionic concentrations interfere with the activity of certain antibacterial peptides, e.g. mammalian defensins (Selsted *et al.*, 1984; Lehrer *et al.*, 1991) or antimicrobial components in haemocytes of the horseshoe crab, *Limulus polyphemus* (Nachum *et al.*, 1980). However, in suspension-based assays, *C. maenas* HLS has strong antibacterial activity in high salt buffers (Chisholm & Smith, 1992; Chisholm, 1993). It is therefore possible that this interference results from interactions between the sample and the agarose at high ionic strength. Accordingly, clear zones were observed when the agarose underlay was prepared with low-salt phosphate buffer. The presence of diluted nutrients in the underlay did not enhance antibacterial activity of crab HLS, as is the case with mammalian defensins (Selsted *et al.*, 1984). Titration of crab HLS in nutrient-free saline buffers has shown that antibacterial activity of HLS is present at total protein concentrations down to about 2 $\mu g\ ml^{-1}$ (Chisholm & Smith, 1992; Chisholm, 1993).

The adaptation of the two antibacterial assays described in this chapter was a prerequisite for the purification of antibacterial factors in *C. maenas* HLS. The next stage, devising a scheme of successive purification steps to separate the antibacterial factors from the bulk of HLS components, is described in the following chapter.

Figure 3.1.1 Titration of crab HLS against *P. immobilis* in the presence or absence of lysozyme. The assay was carried out as described in Materials and Methods. In the lysozyme-free titration, the 10 μl lysozyme stock solution was substituted with 10 μl of CS. The incubation time was 4 hours at 18°C.

Values represent mean $\pm$ SE (n = 3) of the survival indices. Significant differences between the assays (paired t-tests) are indicated (* indicates $p \leq 0.05$).

Figure3.1.1

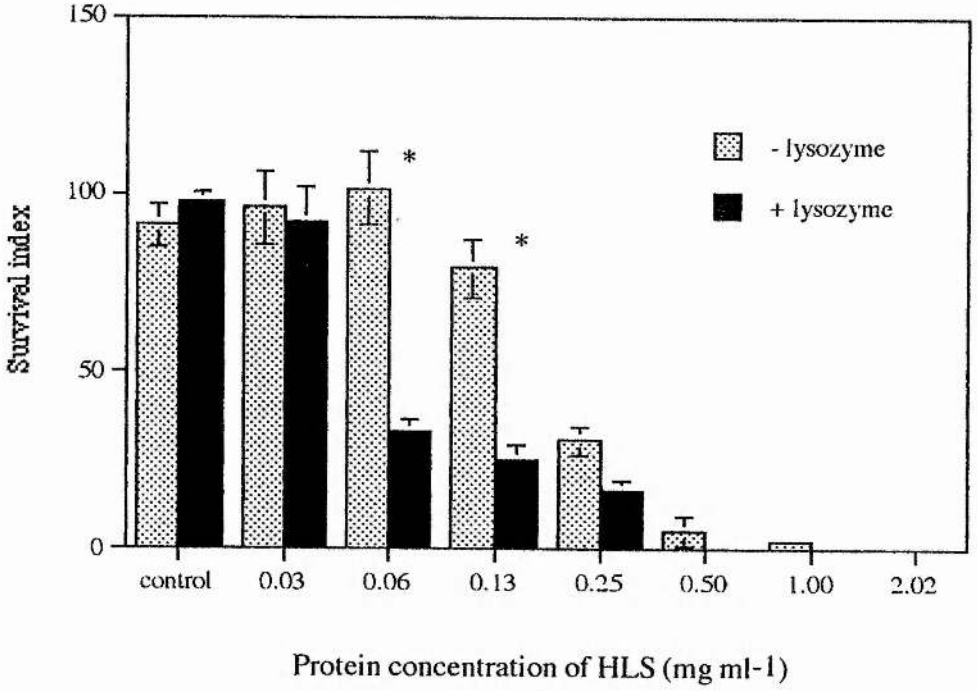

150
100
50
0
Survival index
- lysozyme
+ lysozyme
*
*
control
0.03
0.06
0.13
0.25
0.50
1.00
2.02
Protein concentration of HLS (mg ml-1)

Table 3.1.1. The effect of underlay composition on inhibition zone areas in radial diffusion assays of *C. maenas* HLS against *P. immobilis*.

[1] Underlay composition	[2] Inhibition zone area (mm^2)
Marine broth (undiluted)	0
Marine broth (diluted 1/10 in PB)	24.2 ± 1.5
Marine broth (diluted 1/10 in CS)	1.9 ± 0.1
CS	0
3.2 % NaCl	8.0 ± 0.4
PB	27.2 ± 1.6 **

[1] The underlay consisted of 1% agarose, 0.02% Tween 20 and buffer as shown. It was seeded with ca. 2×10^6 *P. immobilis* ml^{-1}.

[2] Inhibition zone is the area of the clear zone minus the area of the well (ca. 3 mm). Sample volume was 2.5 µl per well. Sample protein content was 0.81 mg ml^{-1} and the end-point titre against *P. immobilis* by spread plate assay was ca. 72.5 µg ml^{-1} in the absence of lysozyme. Values shown are averages ± SE of the mean (n = 3). ** significantly larger inhibition zones than obtained with the other treatments ($p < 0.01$).

Figure 3.1.2. Titration of *C. maenas* HLS against *P. immobilis* using the radial diffusion assay as described in Materials and Methods. The underlay consisted of 1% agarose in PB with 0.02% Tween-20 and ca. 2 x 10^6 bacteria ml^{-1}. Well diameters were 2 mm and sample volumes were 2 µl per well.

Values represent means ± SE (n = 3).

Figure 3.1.2

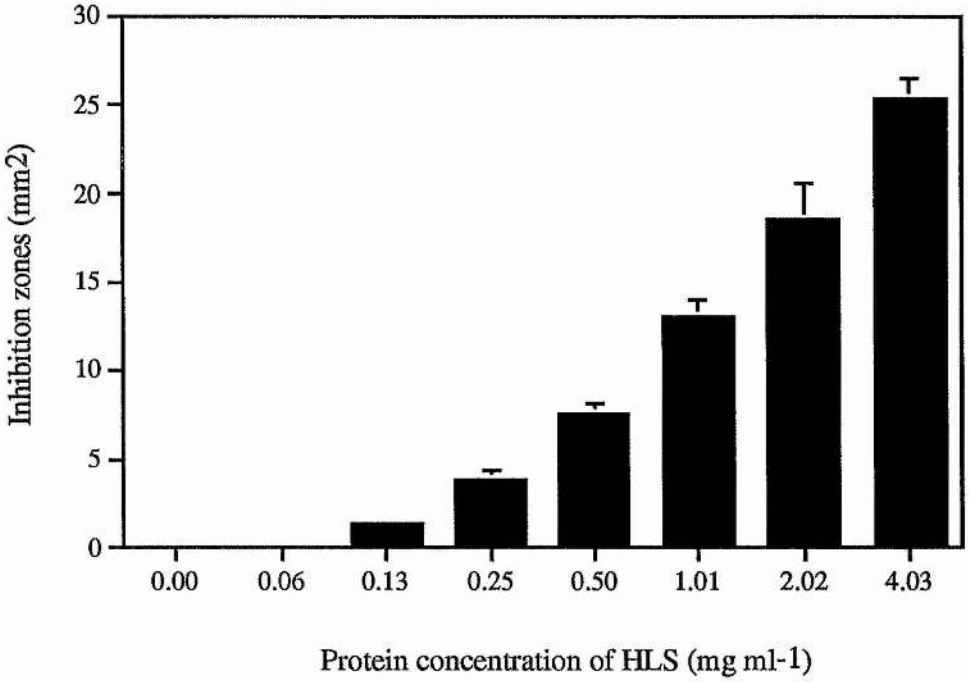

30
25
20
15
10
5
0
Inhibition zones (mm2)
0.00
0.06
0.13
0.25
0.50
1.01
2.02
4.03
Protein concentration of HLS (mg ml-1)

Figure 3.1.3 Effect of dilution of *E. coli* D31 on the sensitivity of the radial diffusion assay: Two-fold dilutions of whole HLS were dispensed into triplicate wells, moving clockwise from the upper right of the petri-dish. Each dish was seeded as follows:

A: ca. 2×10^6 *E. coli* D31 ml^{-1},
B: ca. 2×10^5 *E. coli* D31 ml^{-1},
C: ca. 2×10^4 *E. coli* D31 ml^{-1}.

Conditions were as described in Materials and Methods and the sample used was the same as in Table 3.1.2 (below). The well diameter was 2 mm and the sample volume was 2 μl. Arrows indicate rows with inhibition zones.

Figure 3.1.3

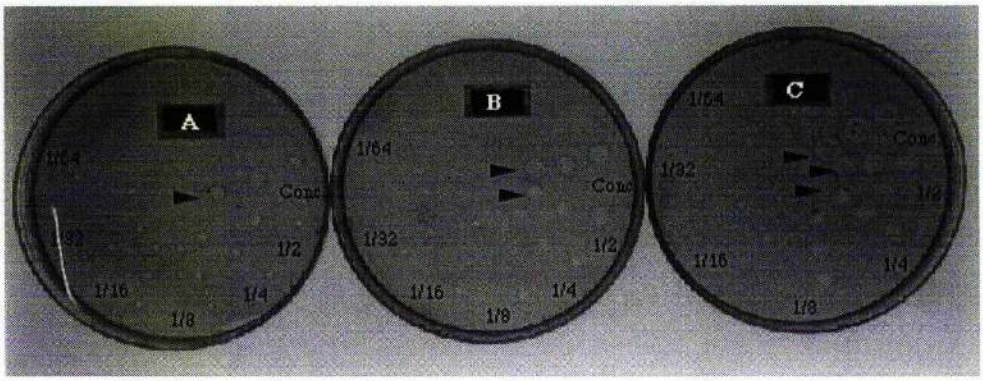


Table 3.1.2. Inhibition zones of *C. maenas* HLS in radial diffusion assays against *E. coli* D21, D22 and D31 at different concentrations of bacteria.

	[1] Inhibition zones (mm^2) at [2] bacterial concentration (ml^{-1})		
Organism	ca. 2×10^6	ca. 2×10^5	ca. 2×10^4
E. coli D21	14.4 ± 1.5	24.8 ± 2.8	35.5 ± 1.3
E. coli D22	35.4 ± 1.3	47.3 ± 0.8	54.8 ± 2.2
E. coli D31	9.0 ± 0.5	18.0 ± 1.4	41.2 ± 2.0

[1] Inhibition zones are areas of clear zones minus area of the wells. The assay was performed as described in Materials and Methods. The well diameter was ca 2 mm and the sample volume was 2 µl. Protein concentration of this sample was 3.37 mg ml^{-1}. Values are means ± SE (n = 3).

[2] Bacterial concentration is the approximate concentration of bacteria ml^{-1} in the agarose underlay.

3.2. SEPARATION OF ANTIBACTERIAL FACTORS IN *C. MAENAS* HLS.

Introduction

Preliminary fractionation of *C. maenas* HLS on Sephadex G-100 by Chisholm (1993) has indicated that there are at least three antibacterial factors in *C. maenas* haemocytes. It was the aim of this chapter to derive a purification schedule for these antibacterial components. At least one of the antibacterial proteins in *C. maenas* HLS appears to be a low molecular weight factor (Chisholm, 1993). Following the definition given by Boman (1995), that antibacterial proteins with a molecular weight of < 10 kDa are peptides, the low molecular weight factor of *C. maenas* will be referred to as a peptide.

P. immobilis was selected as a representative Gram negative test bacterium because of its known susceptibility to antibacterial proteins in *C. maenas* HLS (Chisholm & Smith, 1992; Chisholm, 1993). Two Gram positive test bacteria were also included in this study. *Micrococcus luteus* was selected because of its known sensitivity to antibacterial factors and widespread use in other studies (Boman, 1994). The marine Gram positive bacterium *Planococcus citreus* was included because it is known to be susceptible to antibacterial factors in *C. maenas* HLS (Chisholm & Smith, 1992; Chisholm 1993).

Materials and Methods

Animals and HLS preparation.

Specimens of *C. maenas* were collected and maintained as described in Chapter 2.1. (page 64) and HLS was prepared and assayed for protein as described in Chapter 2.3 (page 110-111). The homogenizing buffers consisted of 0.01% PTU (final concentration) in one of the following: CS for ammonium sulphate

fractionation and calibration of CM Sephadex, deionized water (DW) for SepPak C_{18} extraction and gel filtration or 0.05 M glycine (pH 8.5) for ion exchange chromatography.

Bacteria

P. immobilis, *M. luteus* (NCIMB 376) or *P. citreus* (NCIMB 1493) were maintained and grown as described for *P. immobilis* in Chapter 3.1 (page 130). Absorbances of 0.5 at 570 nm gave bacterial concentrations of ca. 1 x 10^8 ml^{-1} or ca. 5 x 10^7 ml^{-1} for *P. citreus* or *M. luteus* , respectively.

Electrophoresis

Detailed protocols for polyacrylamide gel electrophoresis (PAGE) and the staining of gels are given in Appendix B.

Sodium dodecyl sulphate PAGE under reducing conditions (SDS-PAGE) was carried out according to the method by Schägger & von Jagow (1987), using a 4% stacking gel, 10% spacer gel and a 16.5% separating gel. All buffers were made up in DW. The anode buffer consisted of 0.2 M tris (pH 8.9) and the cathode buffer consisted of 0.1 M tris, 0.1 M tricine and 0.1% (final concentration) SDS (pH 8.2). The separating gel comprised 3.33 ml acrylamide-N,N'-methylene bisacrylamide (Bis) stock solution (1.5 % Bis, 48% acrylamide), 3.33 ml gel buffer (3 M tris, 0.3% SDS, pH 8.45), 2.27 ml DW, 1.07 ml glycerol, 5 µl N,N,N',N',-tetramethylethylenediamine (TEMED) and 50 µl 10% ammonium persulphate (APS). The spacer gel comprised 1.53 ml acrylamide stock solution, 2.5 ml of gel buffer, 3.5 ml of DW, 2.5 µl TEMED and 25 µl APS. The stacking gel consisted of 0.5 ml acrylamide stock solution, 1.55 ml gel buffer, 4.2 ml DW, 7.0 µl TEMED and 70 µl APS. These amounts were sufficient to run two minigels on a Mini

Protean II electrophoresis unit (Bio-Rad, Hemel Hempsted, Hertfordshire). Prior to electrophoresis, samples and molecular weight markers were added to an equal volume of double strength sample buffer (4 ml 20% SDS, 2.4 ml glycerol, 0.4 ml 2-mercaptoethanol, 2 ml 0.5% bromophenol blue, 1 ml 0.1 M tris, pH 6.8) and incubated for 40 min in a waterbath at 60°C. Gels were run at 35 mA until the sample had entered the stacking gel, then at 50 mA until the dye-front had reached the bottom of the gel, typically after 1.5 h. Gels were fixed and stained simultaneously overnight in 0.05% Coomassie brilliant blue R (Sigma) in 45% methanol, 10% glacial acetic acid (final concentrations) and destained in 10% glacial acetic acid.

Molecular weight (MW) markers used for SDS-PAGE were either broad range MW markers from Bio-Rad or Sigma MW-SDS-17S molecular weight markers. The Bio-Rad broad range markers consisted of myosin (200.0 kDa), β-galactosidase (116.2 kDa), phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). The Sigma MW-SDS-17S molecular weight markers consisted of myoglobin polypeptide backbone residues 1-153 (16.95 kDa), myoglobin fragments I+II (residues 1-131, 14.44 kDa), myoglobin fragments I+III (residues 56-153, 10.60 kDa), myoglobin fragment I (residues 56-131, 8.16 kDa), myoglobin fragment II (residues 1-55, 6.21 kDa), glucagon (3.48 kDa) and myoglobin fragment III (residues 132-153, 2.51 kDa). Occasionally, BSA (66.2 kDa), ovalbumin (45.0 kDa) or chymotrypsinogen (25.0 kDa) (all from Pharmacia) were included with the Sigma MW-SDS-17S markers. The molecular weight of proteins on the separating gel was determined empirically from equations fitted to plots of log MW of the markers against their relative mobility (Rf) (Hames & Rickwood, 1981).

Native PAGE under acidic conditions was carried out according to the method of Lehrer *et al.* (1991) using continuous gels containing 12% acrylamide. The running buffer consisted of 5% glacial acetic acid. The gels were composed of 6.4 g of urea, 9.0 ml of DW, 0.8 ml of 10% APS, 5.33 ml of a solution of 43.2% glacial acetic acid and 4% TEMED and 3.81 ml of acrylamide-Bis stock solution (60% acrylamide, 1.6% Bis). This amount was sufficient for two minigels. Gels used for native acid PAGE were pre-run for 1 h at 150 V constant voltage with reversed polarities to remove the APS and TEMED. Samples were prepared by adding an equal volume of double-strength sample buffer (6 M urea, 10% glacial acetic acid, 0.1% methyl green) and electrophoresed under the same conditions until the green component of the dye front had migrated past the bottom of the gels (ca. 45 min). The gels were stained and destained as described above.

Antibacterial assays

Spread-plate assays were used to assess the antibacterial activity of fractions obtained from the successive precipitation of antibacterial proteins in *C. maenas* HLS with ammonium sulphate (AS) or the pH calibration of CM-Sephadex. The pH calibration of CM-Sephadex was carried out at the beginning of the study when no alternative antibacterial assays were used. To investigate whether or not the synergistic effect of lysozyme (Chapter 3.1, page 135) is due to a specific protein, *C. maenas* HLS fractionated by successive AS precipitation was incubated both in the presence and absence of lysozyme. The assays, using *P. immobilis* as sentinel organism, were carried out as described in Chapter 3.1 (page 131-132), except that antibacterial activity was expressed as percentage survival after 4 h incubation compared to the control, not the SI.

In later experiments, radial diffusion assays (Lehrer *et al.*, 1991) were used because they required smaller sample volumes than spread-plate assays, thus HLS fractions could be assessed for antibacterial activity against several strains and

sufficient material could be retained for SDS-PAGE and protein determination. Radial diffusion assays were used for gel filtration, cation exchange chromatography and extraction of *C. maenas* HLS on SepPak C_{18} cartridges. They were carried out as described in Chapter 3.2 (page 131-132), except that the underlay consisted of 1% agarose and 0.02% Tween-20 (final concentrations) in NaCl-free phosphate buffer (PB). Bacterial concentrations were ca. 2 x 10^6 ml^{-1} in case of *P. immobilis* and ca. 1 x 10^5 ml^{-1} in case of *M. luteus*. A negative control consisted of the buffer in which the sample was suspended. a positive control consisted of 2 µl of whole HLS which had been frozen in 50 µl aliquots for use as standard in antibacterial assays and inclusion in SDS-PAGE.

Antibacterial overlays of native acid PAGE gels on lawns of *P. immobilis* or *Planococcus citreus* were modified from the method by Lehrer *et al.* (1991). After electrophoresis, the gels were washed for 15 min in 150 ml PB, to which 200 µl 1.0 M NaOH had been added, and then washed twice for 10 min in PB alone. They were then placed onto a lawn pre-seeded with ca. 2 x 10^6 bacteria ml^{-1} in 1% agarose and 0.02% Tween 20 (final concentrations) in PB (lawn thickness ca. 1.2 mm). After 1 h incubation at 18°C, the gels were removed and stained as described above. The bacterial lawns were overlaid with an equal volume of double strength marine broth (Difco) in 1% agarose and incubated at 25 °C for 24 h until clear zones appeared. They were stained overnight with a formaldehyde staining solution consisting of 2 mg Coomassie brilliant blue R (Sigma), 27 ml methanol, 63 ml DW and 15 ml 37% formaldehyde (Lehrer *et al.*, 1991) .

Gels and lawn overlays were photographed or sealed in plastic bags, scanned and documented with NIH-Image analysis software for Macintosh (National Institutes of Health, Bethesda, Washington DC, USA).

Gel permeation chromatography

For analytical gel filtration, 1 ml of HLS was applied to a 76 x 1.0 cm column of Sephadex G75 (Pharmacia, Uppsala, Sweden), pre-equilibrated with 0.1 M ammonium acetate, containing PTU at a final concentration of 0.01%, and calibrated with dextran blue and molecular weight markers: BSA (66.2 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and RNAse A (13.7 kDa) (all from Pharmacia). The sample was eluted at a flow rate of 6 ml h^{-1}. One millilitre fractions were collected and the absorbance monitored at 280 nm. Fractions were concentrated by freeze-drying, reconstituted in 100 µl sterile 3.2 % NaCl (pH 7.2) and subjected to antibacterial assays against *P. immobilis* or *M. luteus* using the radial diffusion method. Ten microlitres from every third fraction were also subjected to SDS PAGE.

Successive ammonium sulphate precipitation

Successive precipitation of *C. maenas* HLS with 20%, 40%, 60% and 80% saturation of AS was carried out according to Harris (1989): 2.5 ml of HLS were made up to a volume of 10 ml with DW. An aliquot of 100 µl was removed and kept at -20°C for use in antibacterial assays against *P. immobilis*. Ammonium sulphate was dissolved in the remaining sample to a saturation of 20% (1.07 g total). After incubation on ice for 1 h, the sample was centrifuged at 10, 000 *g* for 15 min at 4°C. The supernatant was transferred to a fresh tube and ammonium sulphate was added successively to give saturations of 40% (1.15 g), 60% (1.22 g) and 80% (1.31 g), followed each time by incubation on ice for 1 h and centrifugation at 10, 000 *g* for 15 min. After each precipitation, the pellets were resuspended in 1 ml distilled water, and transferred, together with 1 ml from the supernatant of the 80% fraction, to benzoylated cellulose dialysis tubing with a nominal molecular weight limit (NMWL) of 2000 Da (Sigma). The samples were dialysed against 500 ml distilled

water for 3 h with one buffer change. Aliquots of 200 µl were removed for protein determination assay of antibacterial activity. Each sample was diluted ten-fold in sterile 3.2% NaCl and assayed for activity against *P. immobilis* using the spread-plate method with and without lysozyme.

For analysis by SDS-PAGE, HLS was successively precipitated with 30% AS (1.66 g per 10 ml) and 90% AS (5.45 g per 10 ml) as described above.

Extraction on Sep Pak C_{18} cartridges

A Sep Pak C_{18} cartridge (Whatman, Maidstone, Kent) was washed with 4 ml methanol and 4 ml 0.1% trifluoroacetic acid (TFA) in DW. Two millilitres of HLS, prepared in DW containing a final concentration of 0.01% PTU, was acidified by adding TFA to a final concentration of 0.1% and applied to the cartridge. After washing with 3 ml 0.1% TFA, the proteins were eluted with 2 ml each of a step-gradient of 20%, 40%, 60% or 80% acetonitrile (ACN) in 0.1% TFA. The flow rates were kept below 2 ml min^{-1}. Fractions of 1 ml were collected manually and TFA and ACN removed by freeze-drying. Fractions were reconstituted in 500 µl of DW and subjected to assays of protein concentration, antibacterial activity against *P. immobilis* or *M. luteus* , using the radial diffusion assay as described in Chapter 3.1 (page 131-132) and SDS-PAGE.

Cation exchange chromatography

The cation exchange resin (CM-Sephadex, Pharmacia) was calibrated according to pH as described by Roe (1992). Ten test tubes were set up, each containing 250 µl of CM-Sephadex pre-swollen in DW. The gel in each tube was washed with ten 10 ml volumes of 0.5 M and five of 10 ml volumes of 0.05 M of one of the following buffers: ammonium acetate/acetic acid (pH 5.0 to 5.5),

ammonium formate/formic acid (pH 6.5 to 7.5) or glycine/NaOH (pH 8.5 to 10.5). The pH values ranged from 5.0 to 10.5 at intervals of 0.5 pH units. After washing, 200 µl of HLS in DW, containing 0.01% PTU (final concentration), were added to each tube. The tubes were left to mix on an orbital shaker for 30 min at 18°C. One hundred microlitres of the supernatant from each gel, together with 100 µl of each buffer (at 0.05M concentration) were removed and an equal volume of sterile, tris-buffered 6.4% NaCl (0.05 M tris, pH 7.2) added prior to assays of antibacterial activity against *P. immobilis*. Samples were assayed by the spread-plate method without lysozyme.

After determination of the running pH, a 5 ml column of CM Sephadex was equilibrated with 0.05 M glycine, pH 8.5. Two millilitres of HLS, prepared in 0.05 M glycine, pH 8.5, containing a final concentration of 0.01% PTU, were applied to the column. The proteins were eluted with a linear gradient of 0.0 M to 0.5 M NaCl in 0.05 M glycine, pH 8.5, without PTU. The flow-rate was 1 ml min^{-1}. One millilitre fractions were collected, assayed for protein and antibacterial activity against *M. luteus* or *P. immobilis* by radial diffusion assay and subjected to SDS-PAGE.

Statistical analysis

The effect of lysozyme on antibacterial activity of *C. maenas* HLS, fractionated by successive ammonium sulphate precipitation, was analysed with unpaired t-tests (Campbell, 1990), because separate controls were used to determine the percentage of bacterial survival for each treatment. Comparisions of antibacterial activity between fractions was not possible because the variation of antibacterial activity with protein concentration could not be quantified, as more than one antibacterial protein may have been present. An overall correlation of antibacterial activity with protein content of precipitated fractions was determined using

Kendall's signed rank test (Campbell, 1990). A probability level of ≤ 0.05 was accepted as significant.

The pH-calibration of CM-Sephadex for cation exchange of *C. maenas* HLS was set up as a qualitative test. Because protein content of the supernatant increases with increasing pH, as fewer proteins bind to the resin, a correlation of antibacterial activity with protein content was expected. This was tested using Kendall's signed rank test (Campbell, 1990), with a significance level of $p \leq 0.05$.

The radial diffusion assay allows quantification of specific activity (inhibition zone area divided by protein content), of an antibacterial protein characterized by titration (Hultmark *et al.*, 1982). However, if several antibacterial proteins are present in a sample in undefined ratios, quantitative comparision of specific activity is not possible (Dimarq *et al.*, 1987). In *C. maenas* HLS extracted on a Sep Pak C_{18} cartridge, the correlation of antibacterial activity with protein content was instead analysed by Kendall's signed rank test (Campbell, 1990), accepting a significance level of $p \leq 0.05$. No statistical comparisions were carried out between fractions of *C. maenas* HLS separated by column chromatography.

Results

Antibacterial overlays of native acid PAGE gels

Acid native PAGE gels of whole *C. maenas* HLS, together with their overlays on lawns of *P. immobilis* or *P. citreus,* are shown in Figure 3.2.1. At least four clear zones are present in the overlay on *P. citreus*, and three clear zones are present in the overlay on *P. immobilis* (Figure 3.2.1).

Gel permeation chromatography

An elution profile of HLS from a column of Sephadex G-75 is shown in Figure 3.2.2, together with the profiles of activity against *P. immobilis* or *M. luteus* and SDS-PAGE analysis of fractions.

Activity against *P. immobilis* is present in most fractions, especially fractions 27-36, 38-40 42-56 and 57-65 (Figure 3.2.2 c). By contrast, activity against *M. luteus* occurs in fractions 43-55, 58-59 and 63-67 (Figure 3.2.2 c).

The positions of the molecular weight markers, shown above the elution profile, indicate that the main antibacterial activities correspond to proteins of < 25 kDa, although antibacterial activity is also present in protein fractions eluted at ca. 45 kDa and >70 kDa (Figure 3.2.2 b). The SDS-PAGE profiles of fractions 25-37 contain numerous bands (Figure 3.2.2 a). It was not possible to determine which of these represent proteins with antibacterial activity. From fraction 37, the elution of a prominent band of ca. 11.6 kDa becomes evident (Figure 3.2.2 a). A protein of ca 20 kDa is present in SDS-PAGE profiles of fractions 43-52 and from fraction 46 onwards, a peptide of ca. 6.5 kDa appears in the SDS-PAGE profiles (Figure 3.2.2 a). Bands with molecular weights of less than 6.5 kDa were not eluted in the remaining part of the profile. Instead, a protein of ca. 25 kDa, which may have interacted with the gel or glass of the column, is present in fraction 61 (Figure 3.2.2 a). The antibacterial activity against *P. immobilis* and *M. luteus* in fractions 42-61 (Figure 3.2.2 c) is thus associated with peptides and proteins with molecular weights of ca. 6.5 kDa, 11.6 kDa, 20 kDa or 25 kDa.

Ammonium sulphate precipitation

Table 3.2.1. shows the percentage survival of *P. immobilis* after 4 h incubation with ten-fold diluted *C. maenas* HLS or fractions obtained from successive AS precipitations. Whole HLS (0% AS) showed 100% killing of *P. immobilis* (0 % survival) (Table 3.2.1). The 20% AS fraction showed virtually no antibacterial activity in the absence of lysozyme (82.02% ± 7.75% survival), although the percentage of bacterial survival was significantly reduced in the presence of lysozyme (1.65% ± 1.14% survival) (Table 3.2.1). Most of the protein precipitated at 40% AS saturation and antibacterial activity in this fraction was also strongest, although it was not significantly higher in the presence of lysozyme (1.51% ± 1.38% survival with lysozyme or 5.04% ± 2.22% survival without lysozyme, $p > 0.05$) (Table 3.2.1). Bacterial survival in the 60% AS fraction was again significantly reduced in the presence of lysozyme (3.5% ± 1.5% or 10.5% ± 2.5% in the presence or absence of lysozyme, respectively, $p < 0.02$) (Table 3.2.1). Killing was evident neither in the 80% AS fraction nor the supernatant retained from the 80% AS fraction (Table 3.2.1). Fractions of *C. maenas* HLS obtained by successive ammonium sulphate precipitation did not show a correlation between protein content and antibacterial activity (Kendall's signed rank statistic, $p = 0.33$ for sampls without lysozyme). Statistical analysis did not indicate that the synergistic effect of lysozyme with antibacterial factors in *C. maenas* HLS is due to one specific protein, which could be cut by AS precipitation.

Figure 3.2.3. shows SDS-PAGE profiles of successive precipitations of *C. maenas* HLS with 30% and 90% AS. Differences between the two profiles are indicated by arrows. The calibration of the gel is shown in Figure 3.2.4. From the equation fitted to the plot (Figure 3.2.4), the molecular weights of proteins present in the 30% AS fraction, but not the 90% fraction (Figure 3.2.3), were calculated to be ca. 40.6 kDa and 6.7 kDa, respectively. The molecular weights of proteins present in

the 90%, but not 30% fraction (Figure 3.2.3), were calculated to be ca. 61.6, 26.7 and 20.0 kDa, respectively.

Sep Pak C_{18} extraction

Table 3.2.2 shows antibacterial activity and protein content of *C. maenas* HLS extracted on a Sep Pak C_{18} cartridge in the presence of a step-gradient of ACN. Antibacterial activity was present in all fractions, but it was strongest in the 40% fraction, where inhibition zones of 59.1 $\pm$ 1.9 mm^2 were obtained for *P. immobilis*, and 59.9 $\pm$ 0.5 mm^2 were obtained for *M. luteus* respectively (Table 3.2.2). This contrasts with inhibition zones in the flow-through of 36.1 $\pm$ 3.4 mm^2 for *P. immobilis*, and 15.27 $\pm$ 1.97 mm^2 for *M. luteus* (Table 3.2.2). The protein content was considerably higher in the flowthrough than in the 40% fraction (6.10 $\pm$ 0.03 mg ml^{-1} and 0.57 $\pm$ 0.04 mg ml^{-1}, respectively) (Table 3.2.2). The protein content of the fractions did not correlate with antibacterial activity (Kendall's signed rank statistic, $p = 0.23$).

The SDS-PAGE profile of a Sep Pak C_{18} extraction of *C. maenas* HLS with 0%, 10%, 20%, 50% or 80% ACN shows several protein bands, the most prominent of which is an 11.6 kDa protein (Figure 3.2.5). Two bands, representing proteins of molecular weights of ca. 67.7 kDa and 83.1 kDa respectively, elute at ACN concentrations of up to 50% and a band of ca. 41.6 kDa is present throughout the elution profile (Figure 3.2.5).

Cation exchange chromatography

Table 3.2.3 shows the pH calibration of CM-Sephadex for cation exchange chromatography of antibacterial factors in *C. maenas* HLS. Strong antibacterial activity, indicated by low percentages of bacterial survival (< 5%), is present at

pH 6.5 to 10.5, showing that one or more antibacterial factors do not bind to the cation exchange resin at near neutral pH (Table 3.2.3). Activity is strongest at pH 9.5 to 10.5 (all 0 % survival) (Table 3.2.3), showing the presence of cationic antibacterial factors which remain bound to the column up to a pH of 9.5. Protein content of the supernatants tended to increase with increasing pH, from 0.24 mg ml^{-1} at pH 5.5 to 0.41 mg ml^{-1} at pH 10.5 (Table 3.2.3) and antibacterial activity was found to correlate with protein content (Kendall's signed rank test, $p < 0.01$). The percentage survival of *P. immobilis* in the buffer controls was between 82% and 100% for all buffers, except for glycine at pH 10.5 where it was 77.9% (Table 3.2.3).

Based on the data in Table 3.2.3, a pH of 8.5 was chosen for cation exchange of *C. maenas* HLS on a column of CM-Sephadex. Figure 3.2.6. shows the elution profile, antibacterial activity and SDS-PAGE profile of HLS separated in the presence of a 0.0-0.4 M gradient of NaCl, run over 40 minutes. The largest absorbance peak at 280 nm was present in fractions 2-11 representing the flow-through prior to the NaCl gradient, beginning with fraction 11 (Figure 3.2.6 b). This peak was associated with weak antibacterial activity mainly against *P. immobilis* (Figure 3.2.6 c). The biggest inhibition zone of the flow-through is 6.2 mm^2 in fraction 8 (Figure 3.2.6 c). By contrast, antibacterial activity was strong between fractions 27 and 42 for *P. immobilis* and between fractions 27 and 38 for. *M. luteus*, with inhibition zones of up to 16.4 mm for *P. immobilis* (fraction 31) or 14.1 mm for *M. luteus* (fraction 33) (Figure 3.2.6 c). SDS-PAGE analysis of the absorbance peaks in fractions 2-11 and 23-27 and the activity peak in fractions 27-38 are shown in Figure 3.2.6 a. The flow-through, at fraction 8, contains most of the proteins found in *C. maenas* HLS, with the exception of a protein at ca. 6.5 kDa which is present in whole HLS but not the flow-through (Figure 3.2.6 a). The second absorbance peak, at fraction 23, contains some proteins with molecular weights of >17 kDa, which are also present in the flow-through, and a prominent protein band

of ca. 11.6 kDa (figure 3.2.6 a). The profile of the antibacterial activity peak, at fraction 33, contains two bands, one representing a protein of ca 11.6 kDa and one representing a peptide of ca. 6.5 kDa. This 6.5 kDa band, therefore, represents an antibacterial peptide acting either on its own or in conjunction with the 11.6 kDa factor.

Discussion

Overlays of acid native PAGE gels on lawns of *P. immobilis* show that there are at least three factors with activity against Gram negative bacteria in *C. maenas* HLS. Overlays on lawns of *P. citreus* further show that there are at least four factors which are active against this Gram positive bacterium. However, from gel permeation chromatography of *C. maenas* HLS, it is apparent that activity against the Gram positive strain, *M. luteus*, was less pronounced than activity against the Gram negative strain, *P. immobilis,* and is mainly restricted to the low molecular weight components.

SDS-PAGE analysis of the gel filtration profile shows that one or more of the antibacterial proteins in *C. maenas* HLS fall into the molecular weight ranges of ca. 6.5, 11.6, 20.0 or 25.0 kDa. Cation exchange chromatography on a CM-Sephadex column resulted in the partial separation of a 6.5 kDa peptide from an 11.6 kDa protein with which it was co-eluting, and this is associated with pronounced antibacterial activity against both *P. immobilis* and *M. luteus*. This is an indication that a 6.5 kDa cationic peptide is one of the factors responsible for the antibacterial activity in *C. maenas* HLS, although it may be acting in conjunction with the 11.6 kDa factor or other haemocyte components.

Previously, no antibacterial factors of this size have been reported for the invertebrates. Most of the antibacterial peptides isolated from insects, including the defensins and cecropins, have molecular weights of ≤ 4 kDa (Cociancich *et al.*, 1994a; Hara & Yamakawa, 1995; Levashina *et al.*, 1995; Chernysh *et al.*, 1996). By contrast, coleoptericin (from *Zophobas atratus*), diptericin (from *Phormia terranovae*) and hymenoptaecin (from *Apis mellifera*) have molecular weights between 8 and 10 kDa, attacins (from *Hyalophora cecropia*) have molecular weights of 20 kDa and sacrotoxins II (from *P. terranovae*) have molecular weights of 24 kDa (Cociancich *et al.*, 1994). Tachyplesins and anti-LPS factor from horseshoe crabs have molecular weights of around 2.5 and 10 kDa, respectively (Iwanaga *et al.*, 1994). Recently, a big defensin (a hybrid molecule combining a hydrophobic region at the N-terminal with a defensin-like region at the C-terminal) has been described from *Tachypleus tridentatus* (Saito *et al.*, 1995). This molecule has an MW of about 8 kDa and is therefore larger than the *C. maenas* 6.5 kDa peptide.

One aim of the work presented in this chaper was to develop a purification scheme for antimicrobial factors in crab HLS. A prerequisite for protein or peptide purification is the determination of some properties of the target molecule which can be exploited in successive separation steps (Harris, 1989a). The techniques investigated here included separation by size (gel permeation chromatography), hydrophobicity (ammonium sulphate fractionation and Sep Pak C_{18} extraction) and charge (cation exchange chromatography). Gel permeation chromatography was of limited use for the preliminary purification of HLS factors because antibacterial activity eluted throughout the profile. However, by focussing on factors within the lower molecular weight range, it was possible to obtain relative pure fractions containing only traces of proteins with MWs > 25 kDa. The disadvantage of this method is that it is relatively time-consuming and leads to sample dilution as a result of relatively poor resolution, necessitating the use of long columns and slow flow rates (Prenata, 1989). However, separation according to size is a powerful method

and gel filtration is often used during later stages of purification schemes when target molecules are easier to identify (Harris, 1989a).

A more commonly used method in preliminary protein purification is the selective concentration of target molecules by ammonium sulphate fractionation (Harris, 1989b). However, because several antibacterial factors are present in *C. maenas* HLS, considerable overlap between fractions is to be expected. SDS-PAGE analysis indicates that the precipitate obtained from adding ammonium sulphate to 30% saturation already contains most of the proteins present in HLS. It is therefore concluded that this method would not be suitable for the preliminary purification of antibacterial factors in crab HLS.

In the present study, fractionation of *C. maenas* HLS on a Sep Pak C_{18} cartridge in the presence of ACN was associated with few bands on SDS-PAGE gels, but relatively strong antibacterial activity. This method may thus be better for the separation of antibacterial factors from *C. maenas* HLS. Because it is desirable to exploit as many different properties of the target molecules as possible during the purification scheme (Harris, 1989a), it was felt that this purification step should be retained.

Among the methods investigated in the present study, cation exchange chromatography has emerged as most powerful for separating antibacterial factors in *C. maenas* HLS. Antimicrobial peptides are generally cationic (Hoffmann, 1995) and cation exchange chromatography is widely used in purification schemes for the isolation of antimicrobial peptides from both vertebrates (e.g. Ganz *et al.*, 1985; Selsted & Harwig, 1987; Gennaro *et al.*, 1989; Alberdi *et al.*, 1995) and invertebrates (e.g. Hultmark *et al.*, 1980, 1982, 1983; Dimarq *et al.*, 1988, Matsuyama & Natori, 1988; Nakamura *et al.*, 1988; Lambert *et al.*, 1989; Cociancich *et al.*, 1994b). All these workers have used a pH of 7.3 or less in their

ion exchange protocols, usually with CM based resins. The pH calibration (Roe, 1989) of CM-Sephadex for cation exchange chromatography of *C. maenas* HLS has lead to the use of a pH of 8.5, and this may have improved the separation of the cationic factors. An advantage of ion exchange chromatography is that it can be modified for bench-top applications using shorter columns and NaCl step-gradients. This results in a more concentrated elution of antibacterial factors, due to rapid increase of salt concentration and small column volumes (Roe, 1989). Furthermore, because there is very little elution of proteins in *C. maenas* HLS at low salt concentrations, relatively large volumes of samples can be applied to the resin. This makes this technique relatively suitable for the selective concentration of cationic antibacterial factors in *C. maenas* HLS prior to further purification.

SDS-PAGE profiles of fractionated *C. maenas* HLS obtained during this study show that low performance chromatography methods are unlikely to be sufficient for the purification of *C. maenas* antibacterial proteins. The resolution of higher molecular weight components is generally poor. A problem with larger proteins is the possibility of degradation or loss of activity during lengthy purification procedures and it is not advisable to include more than 3-4 steps in a purification scheme (Harris, 1989a). Much of the antibacterial activity present in *C. maenas* HLS is stable upon heat treatment or freezing for prolonged periods (Chisholm & Smith, 1992). The heat stable component of the activity probably resides in the lower molecular weight factors in *C. maenas* HLS. Thus, it was decided to focus on the low molecular weight proteins. However, the 6.5 kDa peptide and the 11.6 kDa protein were found to share similar properties in terms of size, hydrophobicity and charge. Their separation could not be improved by further alteration of the conditions during cation exchange chromatography (steepness and range of NaCl gradient, running pH), the most powerful chromatography method investigated so far. The two purification schedules to be investigated in the following chapter were therefore decided to include the following steps:

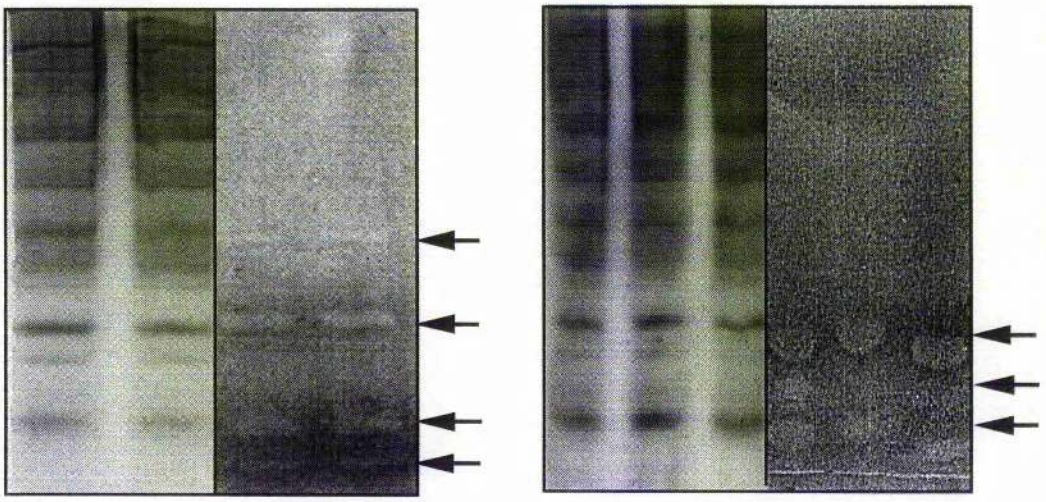
A. SepPak C_{18} extraction, followed by gel filtration and reverse phase HPLC (RP-HPLC);

B. Gel filtration, followed by cation exchange chromatography of low molecular weight fractions and RP-HPLC.

Detailed protocols for these purification schedules are given in Appendix C.

Figure 3.2.1 Acid native PAGE of *C. maenas* HLS and antibacterial overlays on lawns of *P. citreus* or *P. immobilis*. The gels were run as and the overlay carried out as described in Materials and Methods. The gels are shown on the left of each picture and the overlays are shown on the right. Clear zones, showing bands with antibacterial activity, are indicated by arrows.

Figure 3.2.1



P. citreus

P. immobilis

Figure 3.2.2 Gel permeation chromatography of *C. maenas* HLS. One millilitre of HLS was applied to a 1.0 x 76 cm column of Sephadex G-75, equilibrated with 0.1 M ammonium acetate containing 0.01% PTU (pH 6.8), and eluted with the same buffer at a flow rate of 6ml h^{-1}. One millilitre fractions were collected and their absorbance measured at 280 nm. Fractions were freeze-dried, reconstituted in 100 µl CS and assayed for activity against *P. immobilis* or*M. luteus* by the radial diffusion method, as described in Chapter 3.1 (page 133-134), using PB as the underlay buffer. Every third fraction from fraction 25 onward was subjected to SDS-PAGE as described in Materials and Methods and Appendix B.

a) SDS- PAGE profile of fractions. The fraction numbers are indicated above each lane. The profile of whole *C. maenas* HLS is shown on the right, with indications of the positions of MW markers. Protein bands mentioned in the text are indicated by arrows.

b) Elution profile of *C. maenas* HLS. The position of MW markers are indicated above the profile.

c) Antibacterial activity of fractions against *M. luteus* and *P. immobilis*.

Figure 3.2.2

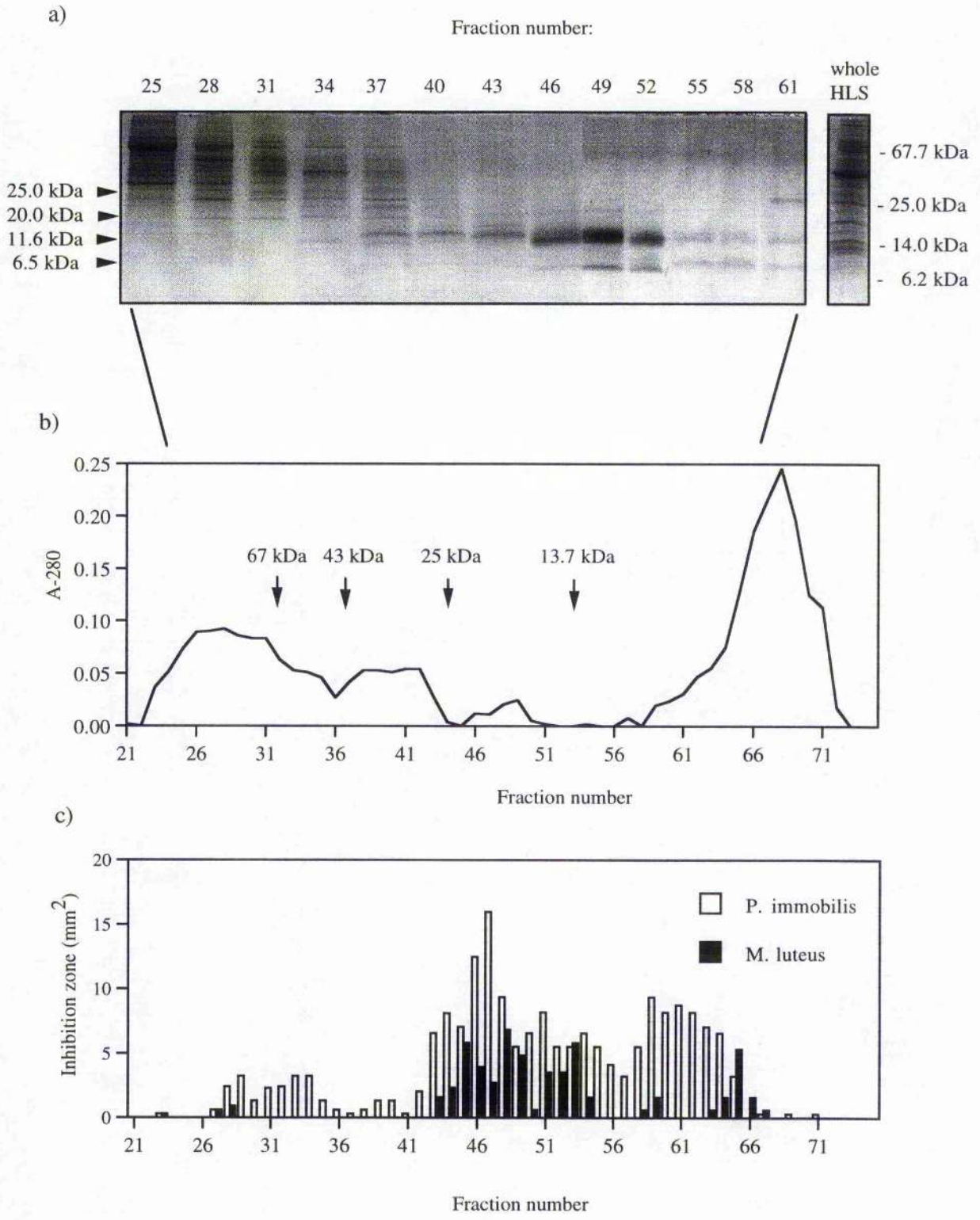

a)
Fraction number:
25 28 31 34 37 40 43 46 49 52 55 58 61
whole HLS
25.0 kDa
20.0 kDa
11.6 kDa
6.5 kDa
- 67.7 kDa
- 25.0 kDa
- 14.0 kDa
- 6.2 kDa
b)
A-280
0.25
0.20
0.15
0.10
0.05
0.00
67 kDa
43 kDa
25 kDa
13.7 kDa
21 26 31 36 41 46 51 56 61 66 71
Fraction number
c)
Inhibition zone (mm2)
20
15
10
5
0
P. immobilis
M. luteus
21 26 31 36 41 46 51 56 61 66 71
Fraction number

Table 3.2.1 Antibacterial activity against *P. immobilis* in ammonium sulphate fractions of *C. maenas* HLS in the presence or absence of lysozyme

[1] AS (% saturation)	[2] Protein concentration (μg ml^{-1})	[3]Survival of *P. immobilis* (%) without lysozyme	[4] with lysozyme
0	87.5	0.0	0.0
20	7.4	86.0 ± 7.8	1.7 ± 1.1 **
40	262.0	5.0 ± 2.2	1.5 ± 1.4 NS
60	38.4	10.5 ± 2.5	3.5 ± 1.5 *
80	7.6	88.4 ± 4.3	89.1 ± 6.2 NS
Supernatant	5.8	99.5 ± 6.5	103.1 ± 5.8 NS

[1] The sample was made up to 10 ml with DW and ammonium sulphate (AS) added successively to the indicated percentage saturation, after removal of the precipitated protein by centrifugation at 10,000 *g* for 15 min at 4°C.

[2] After each precipitation, the pellets were resuspended in 1 ml DW and dialyzed, together with 1 ml of the supernatant of the 80% fraction, as described in Materials and Methods. For antibacterial assays, samples were diluted ten-fold with sterile 3.2% NaCl. Protein concentration refers to μg ml^{-1} of the diluted samples.

[3] Survival refers to percentage of bacteria in the sample after 4h incubation at 18°C, as compared to the control (sterile 3.2 % NaCl). Values are mean ± SE (n = 3).

[4] Lysozyme was added to a final concentration of 1 mg ml^{-1}. Differences between samples with or without lysozyme: ** $p < 0.01$, * $p < 0.05$, NS not significant.

Figure 3.2.3. SDS-PAGE profiles of successive 30% and 90% AS fractions of *C. maenas* HLS. Molecular weights are indicated on the left hand side, differences between the 30% and 90% profiles are pointed out by arrows on the right hand side. Lanes are as follows:

Lane A: molecular weight markers,
Lane B: whole HLS,
Lane C: 30% AS fraction of HLS,
Lane D: 90% AS fraction of HLS.

The molecular weights of bands indicated by arrows have been determined from the graph shown in figure 3.2.4. as ca. 61.5, 40.6, 26.7, 20.0 and 6.7 kDa, respectively.

Figure 3.2.4. Calibration of the SDS-PAGE gel shown in Figure 3.2.3. with molecular weight markers (myoglobin fragments and glucagon, Sigma). The logarithm of the molecular weights of protein bands in SDS-PAGE gel is proportional to their relative mobility (Rf) (e.g. Hames & Rickwood, 1981). The equation fitted to the plot of Rf versus log MW was used to determine the molecular weights of the bands indicated by arrows in Figure 3.2.3.

Figure 3.2.3

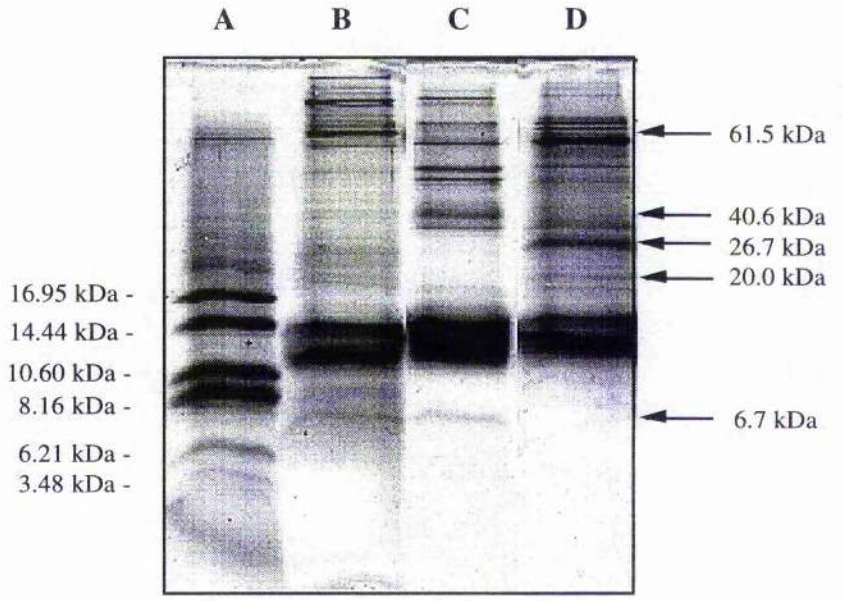


Figure 3.2.4

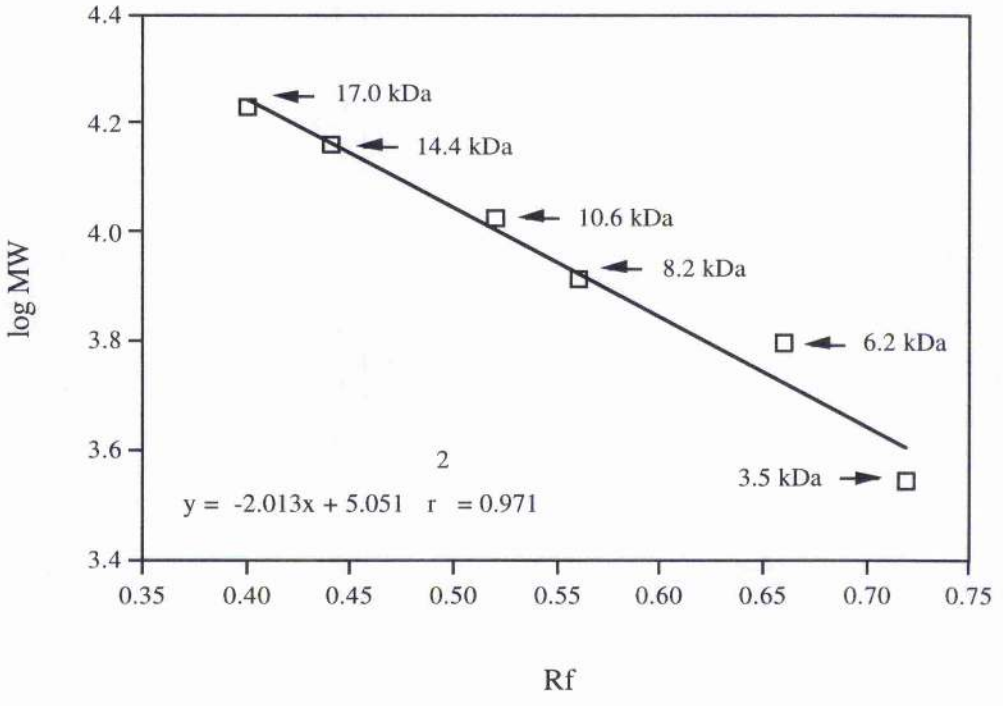


Table 3.2.2. Separation of antibacterial factors of *C. maenas* HLS on a Sep Pak C_{18} cartridge with a step-gradient of ACN

[1] ACN (%)	[2] Protein concentration (mg ml^{-1})	[3] Inhibition zones (mm^2) *P. immobilis*	*M. luteus*
0	6.10 ± 0.03	36.1 ± 3.4	15.3 ± 2.0
20	0.40 ± 0.03	19.3 ± 0.8	14.3 ± 1.2
40	0.57 ± 0.04	59.1 ± 1.9	59.9 ± 0.5
60	0.10 ± 0.05	36.4 ± 1.7	17.1 ± 1.3
80	0.09 ± 0.04	11.5 ± 0.5	4.8 ± 0.9

[1] Each step consisted of 2 ml ACN at the indicated concentration.

[2] Fractions (1 ml) were freeze-dried and reconstituted in 500 µl sterile DW. Values represent means ± SE (n = 3) of the second fraction from each gradient step.

[3] Inhibition zones are from the second fraction of each gradient step. Large zones indicate strong antibacterial activity. Values represent means ± SE (n = 3).

Figure 3.2.5 SDS-PAGE profile of *C. maenas* HLS extracted on a Sep Pak C_{18} cartridge. The gel was run and stained with Coomassie blue as described in Materials and Methods. Protein bands of ca. 83.kDa, 67.7 kDa, 41.6 kDa and 11.6 kDa, mentioned in the text, are indicated by arrows.

Figure 3.2.5

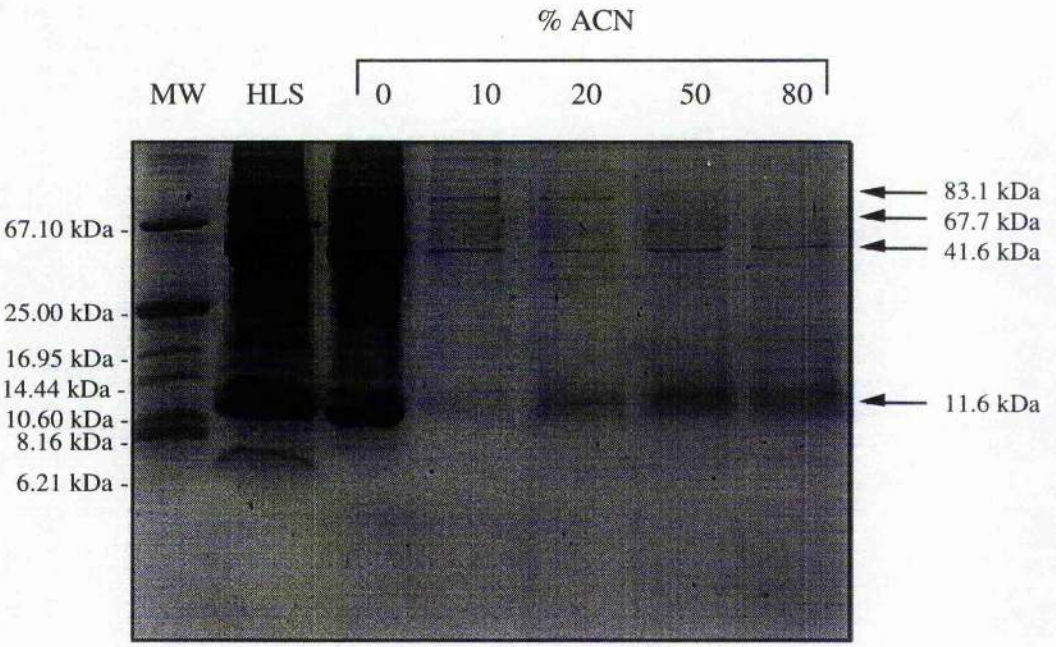

% ACN
MW
HLS
0
10
20
50
80
67.10 kDa
25.00 kDa
16.95 kDa
14.44 kDa
10.60 kDa
8.16 kDa
6.21 kDa
83.1 kDa
67.7 kDa
41.6 kDa
11.6 kDa

Table 3.2.3 Calibration of pH of CM-Sephadex for separating antibacterial factors in *C. maenas* HLS.

[1] Buffer	pH	[2] Protein concentration (mg ml-1)	[3] Survival of *P. immobilis* (%) sample	[4] control
Ammonium acetate	5.5	0.24	51.01	96.64
	6.5	0.32	4.03	100.67
Ammonium	7.0	0.36	2.68	[5] ND
formate	7.5	0.33	2.89	81.89
	8.5	0.38	1.48	96.64
	9.0	0.28	3.36	ND
Glycine-NaOH	9.5	0.37	0.00	89.93
	10.0	0.45	0.00	83.22
	10.5	0.41	0.00	77.85

[1] The resin was washed with buffer as described in Materials and Methods.

[2] Protein concentration of HLS after 30 min incubation with CM-Sephadex and addition of an equal volume of buffered 6.4% NaCl for antibacterial assays.

[3] Percentage of surviving bacteria after 4 h incubation in HLS samples compared to 3.2% NaCl .

[4] Controls consisted of buffers and an equal volume of sterile buffered 6.4% NaCl.

[5] ND = not determined

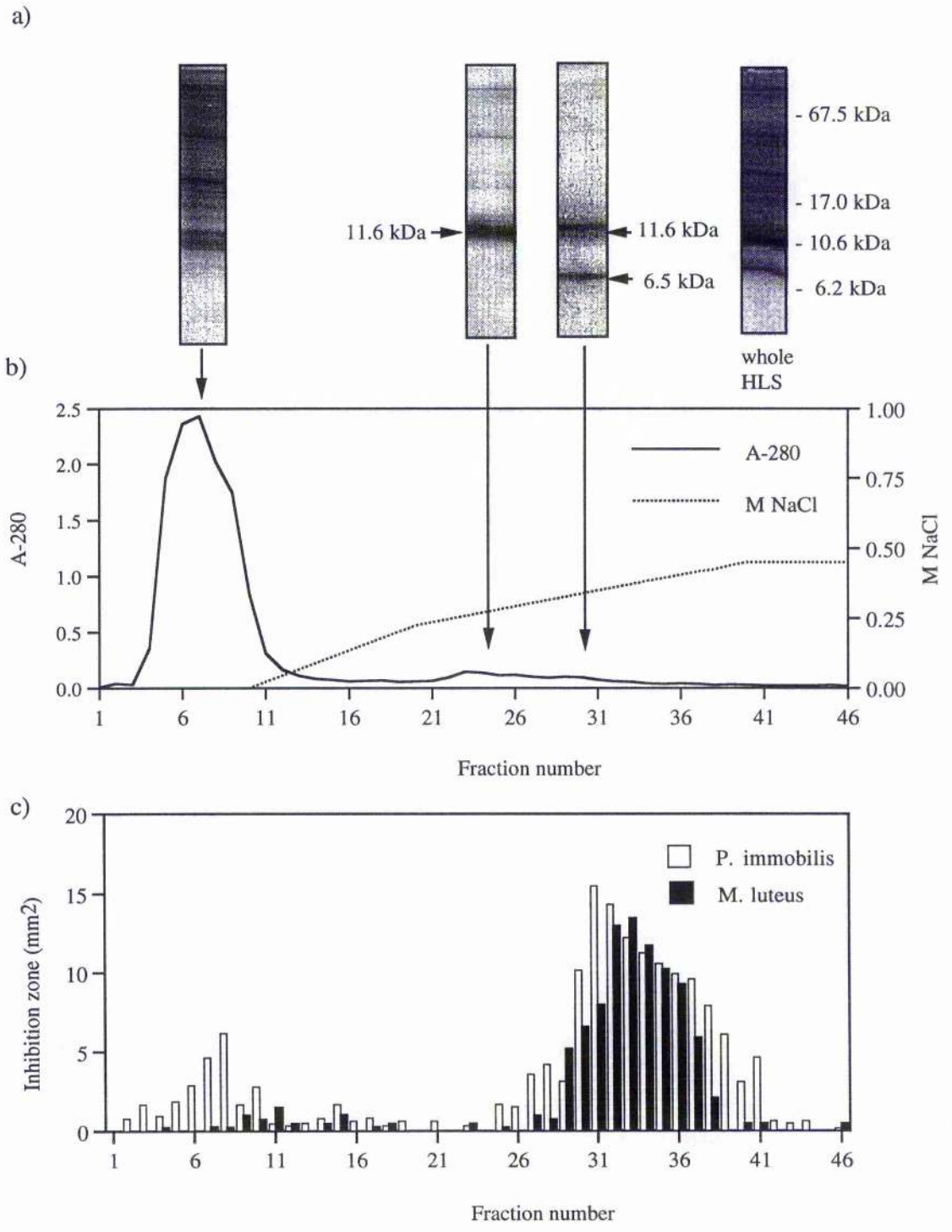
Figure 3.2.6 Cation exchange chromatography of *C. maenas* HLS on CM-Sephadex. Two millilitres of HLS in 0.05 M glycine containing 0.01% PTU (pH 8.5) were applied to a column of CM-Sephadex, equilibrated with the same buffer without PTU and eluted as described in Materials and Methods. One millilitre fractions were collected.

a) SDS-PAGE profiles of absorbance peaks at 280 nm or peaks with antibacterial activity. The SDS-PAGE profile of whole HLS and positions of molecular weight markers are indicated on the right hand side of the figure. Protein bands of ca. 11.6 kDa and 6.5 kDa are indicated by arrows.

b) Elution profile of *C. maenas* HLS. Absorbance was measured at 280 nm.

c) Antibacterial activity of fractions against *P. immobilis* or *M. luteus*. Activity was determined by the radial diffusion method as described in Materials and Methods, using PB as the underlay buffer.

Figure 3.2.6


a)
11.6 kDa
11.6 kDa
6.5 kDa
- 67.5 kDa
- 17.0 kDa
- 10.6 kDa
- 6.2 kDa
whole HLS
b)
A-280
M NaCl
A-280
M NaCl
Fraction number
c)
P. immobilis
M. luteus
Inhibition zone (mm2)
Fraction number

3.3 PURIFICATION OF A 6.5 KDA ANTIBACTERIAL PEPTIDE FROM *C. MAENAS* HLS.

Introduction

In Chapter 3.2, it was shown that *C. maenas* HLS contains several antibacterial proteins, including a hydrophobic, cationic peptide with a molecular weight of ca. 6.5 kDa. Antimicrobial peptides are widespread in nature, and are important for the non-specific host defense in invertebrates and vertebrates alike (Boman, 1995). They have been identified in insects (Hoffman, 1995; Cociancich, 1994), chelicerates (Cociancich et al., 1993; Iwanaga *et al.*, 1994; Saito *et al.*, 1995), amphibians (Barra & Simmaco, 1995) and mammals (Selsted & Oullette, 1995; Boman, 1995; Zanetti *et al.*, 1995), but not so far in the Crustacea. In the present chapter, it will be attempted to purify the *C. maenas* 6.5 kDa peptide to homogeneity and to draw comparisions with antimicrobial peptides described from invertebrates and vertebrates.

The bacteria *P. immobilis* or *M. luteus* were used as Gram negative or Gram positive test organisms, respectively. In addition, *E. coli* D22 was chosen for the titration of antibacterial activity of purified proteins or peptides, because it has been used as standard organism in assays of antibacterial peptides isolated from insects (Boman, 1994) and it is susceptible to antibacterial factors in *C. maenas* HLS (Chapter 3.1, page 134).

Materials and Methods

Animals and HLS preparation.

Specimens of *C. maenas* were collected and maintained as described in Chapter 2.1. (page 64). Haemocyte lysate supernatants were prepared and assayed for protein as in Chapter 2.3 (page 110-111). The homogenizing buffers consisted

of sterile DW, containing 0.01 % PTU (final concentration) .

Bacteria and antibacterial assays

P. immobilis, *M. luteus* or *E. coli* D22 were maintained and grown as described in Chapter 3.1 (page 130) and Chapter 3.2 (page 147). Antibacterial activity was assessed by radial diffusion assay as described in Chapter 3.1 (page 131-132), using PB as underlay buffer. The final concentration of bacteria in the underlay was ca. 2 x 10^6 ml^{-1} for *P. immobilis*, ca. 2 x 10^5 ml^{-1} for *M. luteus*, or ca. 2 x 10^4 ml^{-1} for *E. coli* D22.

Electrophoresis

The method of Schägger & von Jagow (1986) was used for SDS-PAGE under reducing conditions. Electrophoresis was carried out as described in Chapter 3.2 (page 148 - 149). The gels were fixed overnight in 45% methanol, 10% glacial acetic acid (final concentrations) and stained with the Bio Rad silver stain kit. The gels were transferred to oxidiser solution for 15 min, washed 6-7 x in DW over 15 min and exposed to staining solution for 20 min. After washing in DW for 30 s, they were exposed to developer for 30 s and again for ca. 5 min each wash, until a satisfactory contrast was achieved. The staining reaction was stopped with 5% glacial acetic acid (final concentration).

Pre-purification of HLS, Schedule A: SepPak C_{18} extraction and gel filtration

Two millilitres of HLS in sterile DW containing 0.01% PTU (final concentration) were acidified by adding TFA to a final concentration of 0.1% and

applied to a SepPak C_{18} cartridge (Whatman). The cartridge was washed with 3 ml of 0.1% TFA and the proteins eluted with a step-gradient of ACN as described in Chapter 3.2 (page 152). Two fractions of 1 ml were collected for each step of the ACN gradient. Fractions were freeze-dried, reconstituted in 0.5 ml sterile DW and assayed for protein and antibacterial activity against *P. immobilis* or *M. luteus*.

The second of the two fractions eluted with 20% ACN and both fractions eluted with 40% ACN were pooled and 1 ml was applied to a 1.0 x 47 cm column of Sephadex G-75, pre-equilibrated with sterile 0.1 M ammonium acetate (pH 6.8). The column was eluted at a flow rate of 6 ml h^{-1}. One millilitre fractions were collected and absorbance measured at 280 nm. Absorbance peaks were pooled, freeze dried and reconstituted in DW, allowing 50 µl for each ml of original volume. The reconstituted pools were assayed for protein concentration, antibacterial activity against *P. immobilis* and subjected to SDS-PAGE.

Pre-purification of HLS, Schedule B: Gel filtration and ion-exchange chromatography

Gel filtration was carried out as described in Chapter 3.2 (page 151). Eighty microlitres from each fraction, corresponding to numbers 41-54 in Figure 3.2.2 (Chapter 3.2, page 166), were pooled and dialysed against 500 ml DW for 3 h using benzyolated cellulose dialysis tubing with a nominal molecular weight limit (NMWL) of 2 kDa (Sigma). A sample of ca. 1 ml was applied to a 2 ml column of CM-Sephadex, pre-equilibrated in sterile 0.05M glycine (pH 8.5). The column was washed with 5 ml of the equilibration buffer and cationic proteins eluted with 5 ml of 0.05 M glycine, 0.5 M NaCl (pH 8.5). One millilitre fractions were collected manually and analysed directly for protein concentration and antibacterial activity against *P. immobilis* or *M. luteus*. Fractions 10 and 11 were desalted on an ultrafree-

CL filter unit with an NMWL of 5 kDa (Waters) and reconstituted in 200 µl sterile DW.

Comparision of HLS pre-purified by Schedule A or B by analytical reverse phase HPLC

Reverse phase HPLC of partially purified HLS fractions on a microbore C_8 column was carried out by Dr. G.D. Kemp, University of St. Andrews. Pre-purified samples of HLS, subjected to Schedules A or B, were prepared by adding TFA and ACN to final concentrations of 0.1% and 10%, respectively. One hundred microlitres of sample were applied to a microbore C_8 column and eluted with a linear gradient of 25-75% ACN at a flow rate of 80 µl min^{-1}. Eighty microlitre fractions were collected, freeze-dried, reconstituted in 50 µl sterile DW and subjected to assays of antibacterial activity.

Purification of a 6.5 kDa antibacterial peptide for amino acid sequencing

C. maenas HLS was subjected to pre-purification schedule A. The pool containing antibacterial activity was acidified by adding TFA to a final concentration of 0.1%. One hundred microlitres were applied to a Nucleosil C_{18} column, pre-equilibrated with 0.1% TFA, and eluted with a two-step linear gradient of 0-30 and 30-60% ACN at a flow rate of 1 ml min^{-1}. One millilitre fractions were collected, freezed dried, reconstituted in 50 µl DW and assayed for antibacterial activity against *P. immobilis* or *M. luteus*. Ten microlitre aliquots from active fractions were removed and subjected to SDS-PAGE. Fraction 53 was re-chromatographed on the microbore C_8 column as described above. Eighty microlitre fractions were collected and 50 µl aliquots were freeze-dried, re-constituted in 20 µl DW and assayed for activity against *P. immobilis*. The remaining 30 µl were retained for amino acid sequencing. The 6.5 kDa antibacterial peptide was located in fraction 18.

Amino acid sequencing and sequence comparisions

Amino acid sequencing and sequence comparisions were carried out by Dr. G.D. Kemp, University of St. Andrews. Twenty microlitres of fraction 18, obtained as described above, were applied directly to an Applied Biosystems Procise amino acid sequencer (Applied Biosystems, Warrington, Lancashire) and subjected to automated Edman degradation. Sequence comparisions were carried out with the Genetics Computing group (GCG) software package using FASTApep and the Swissprot database, version 29.0.

Titration of antibacterial activity

Antibacterial activity of the purified 6.5 kDa *C. maenas* peptide against *P. immobilis*, *M. luteus* or *E. coli* D22 was titred against antibacterial activity of cecropin A (Sigma) or bovine Bac 7 (gifted by D. Gennaro, University of Trieste, Italy). Cecropins are frequently used as standards to compare antibacterial activity of novel peptides (e.g. Boman, 1994; Chalk *et al.*, 1994), but as they have molecular weights around 4 kDa (Steiner *et al.*, 1981), they are smaller than the *C. maenas* 6.5 kDa peptide. Bac 7 was therefore included as an additional standard. Cecropin A (357.5 μg ml^{-1}), Bac 7 (197.8 μg ml^{-1}) or the *C. maenas* 6.5 kDa peptide (38.9 μg ml^{-1}) were diluted by serial two-fold steps in sterile PB to 1/256, 1/128 or 1/16, respectively. Two microlitres of each peptide dilution were subjected to radial diffusion assays against each bacterium. To minimise variations between zone diameters of replica plates, all three peptides were assayed on the same petri dish; one for each bacterium.

Results

Pre-purification of *C. maenas* HLS

Table 3.3.1 shows the protein concentration and antibacterial activity against *P. immobilis* of pooled fractions of *C. maenas* HLS after extraction on a SepPak C_{18} column, followed by gel filtration (Schedule A). Antibacterial activity is present in pool II, which also contains most of the protein (Table 3.3.1). Some protein, but no antibacterial activity, is present in pools I, III and IV (Table 3.3.1).

Figure 3.3.1 shows the elution profile and antibacterial activity of *C. maenas* HLS, pre-purified by gel filtration and subjected to cation exchange chromatography (Schedule B). Two protein peaks are present in fractions 1-6 and in fractions 9-12 (Figure 3.3.1). The first peak contains antibacterial activity against *P. immobilis*, but not against *M. luteus*. The second peak at fractions 9-12 is associated with stronger activity against *P. immobilis* but also contains activity against *M. luteus* (Figure 3.3.1)

The elution profiles from a microbore C_8 column and antibacterial activity of fractions from HLS, pre-purified by Schedule A or Schedule B, are compared in Figure 3.3.2. In HLS subjected to Schedule A, two absorbance peaks in fractions 16 and 18 (Figure 3.3.2 a) are associated with two activity peaks against both *P. immobilis* and *M. luteus* in fraction 16 and 17-19 (Figure 3.3.2 b). The absorbance peak in fraction 16 is larger (A_{214} ca 0.24) than the peak in fraction 18 (A_{214} ca. 0.09) (Figure 3.3.2 a). By contrast, in HLS subjected to Schedule B the absorbance peak in fraction 16 is smaller (A_{214} ca 0.04) than the peak in fraction 18 ($A_{214} > 0.20$) (Figure 3.3.2 c). The only peak of antibacterial activity against *P. immobilis* and *M. luteus* is associated with the absorbance peak in fraction 18 (Figure 3.3.2 d).

Purification of a 6.5 kDa antibacterial peptide

Figure 3.3.3 shows the purification of the 6.5 kDa peptide from *C. maenas* HLS to homogeneity. Reverse phase HPLC of HLS, pre-purified by Schedule A, on a nucleosil C_{18} column resolved several absorbance peaks at 224 nm (Figure 3.3.3, main graph), two of which had antibacterial activity against *P. immobilis* and *M. luteus* (Figure 3.3.3, histogram). Antibacterial activity against *P. immobilis* was present in fractions 45-50 and in fractions 52-53, whereas activity against *M. luteus* was present in fractions 49-53 (Figure 3.3.3, histogram). Strongest activity against *P. immobilis* was detected in fractions 49 and 53, while that against *M. luteus* was detected in fraction 53 (Figure 3.3.3, histogram). The SDS-PAGE profiles of these fractions are indicated above the main graph in Figure 3.3.3. Two protein bands, at ca. 14 kDa and ca. 11 kDa, are present in the SDS-PAGE profile of fraction 49 and one band, at ca. 6.5 kDa, is present in fraction 53 (Figure 3.3.3, horizontal arrows). The inset to Figure 3.3.3 shows re-chromatography of fraction 53 on a C_8 column. The large peak corresponds to the purified 6.5 kDa peptide (Figure 3.3.3, inset).

Amino acid sequencing and sequence comparisions

A sequence match of the 30 N-terminal amino acids of the 6.5 kDa peptide and bovine Bac 7 (Frank *et al.*, 1990) is shown in Figure 3.3.4. The sequence align shows an overlap of the 30 amino acids of the *C. maenas* 6.5 kDa peptide with 28 amino acids close to the N-terminal end of Bac 7 (Figure 3.3.4). Bold letters indicate identical amino acids (Figure 3.3.4). The overall sequence homology in this overlap is 60.7%.

Titration of antibacterial activity

Figure 3.3.5 compares the antibacterial activity of cecropin A, pure Bac 7 and the purified *C. maenas* 6.5 kDa peptide against *P. immobilis*, *M. luteus*, or *E. coli* D22. Cecropin A is most active against all three strains tested, with end-point titres of 1.7 μg ml^{-1}, 2.6 μg ml^{-1} and < 1.0 μg ml^{-1} against *P. immobilis*, *M. luteus* and *E. coli* D 22, respectively (Figure 3.3.5). The *C. maenas* 6.5 kDa peptide is more active against *P. immobilis* than Bac 7, with end-point titres of 3.7 μg ml^{-1} and 10.0 μg ml^{-1}, respectively, but there is little difference between the activity of the two peptides against *M. luteus* (11.0 μg ml^{-1} and 9.7 μg ml^{-1}, respectively) or .*E coli* D22 (5.6 μg ml^{-1} and 4.7 μg ml^{-1}, respectively) (Figure 3.3.5).

Discussion

Pre-purification of *C. maenas* HLS by Sep Pak C_{18} extraction, followed by gel filtration (Schedule A) yields two antibacterial proteins. By contrast, pre-purification of *C. maenas* HLS by gel filtration and cation exchange chromatography (Schedule B) yields one antibacterial protein. This protein is identical to one of the antibacterial proteins obtained with Schedule A. However, in HLS subjected to Schedule A, the relative amount of this protein is lower. One of the antibacterial proteins obtained from Schedule A consists of either a ca. 11 kDa protein, or a ca. 14 kDa protein. These could not be further separated by reverse phase HPLC. *C. maenas* HLS subjected to either schedule also contained an antibacterial peptide of ca. 6.5 kDa. The sequence of the 30 N-terminal amino acids of this peptide is similar to the sequence of mature bovine Bac 7, described by Frank *et al.* (1990). A 28 amino acid overlap close to the N-terminal end of mature Bac 7 revealed 60.7% homogeneity of the *C. maenas* peptide to Bac 7. The titre of activity of the *C. maenas* 6.5 kDa antibacterial peptide against *M. luteus* and *E. coli* D 22 is also

similar to that of Bac 7, though the *C. maenas* 6.5 kDa peptide is more active against *P. immobilis* than Bac 7. Both peptides show lower titres of activity than cecropin A against *P. immobilis*, *M. luteus* or *E. coli* D 22. However, Cecropin A is smaller (4 kDa) and structurally different, which could have influenced the rate of diffusion, and hence the inhibition zone areas.

The 6.5 kDa antibacterial peptide from *C. maenas* differs in size from any of the antimicrobial peptides described so far for insects (Cociancich *et al.*, 1994a; Boman, 1995; Hoffmann, 1995; Hara & Yamakawa, 1995; Levashina *et al.*, 1995; Chernysh *et al.*, 1996) or horseshoe crabs (*Limulus* spp. and *Tachypleus* spp.) (Iwanaga *et al.*, 1994; Morita *et al.*, 1985; Nakamura *et al.*, 1988; Saito *et al.*, 1995). However, linear proline-rich antibacterial peptides are known to be produced by several insect species, chiefly bees, *Apis mellifera*, the fruit fly, *Drosophila melanogaster,* the silkworm, *Bombyx mori*, and the hemiptera, *Pyrrhocoris apterus* and *Palomena prasina* (Casteels *et al.*, 1989, 1990; Bulet *et al.*, 1993; Cociancich *et al.*, 1994b; Hara & Yamakawa, 1995; Levashina *et al.*, 1995; Chernysh *et al.*, 1996). With the exception of the bee abaecins, silk worm lebocins and *Drosophila* metchnikowins, which are ca. 4 kDa in size, all of these peptides are ca. 2 kDa in size. Amongst these peptides, bee apidaecins show overlap of two proline residues and a PRPP motif with the *C. maenas* peptide, representing 33 % identity with the partial sequence of the latter. The presence of proline residues and the PRPP motif at these positions may be of functional importance, although unlike the *C. maenas* 6.5 kDa peptide, apidaecins are active only against Gram negative bacteria (Casteels *et al.*, 1989). The metalnikowins I, IIA and IIB of the bug *P. prasina* (Chernysh *et al.*, 1996) also share a PRPP motif with the *C. maenas* peptide, whereas metalnikowin III (Chernysh *et al.*, 1996) shares a PRP motif. Overall, the metalnikowins share 20-26 % identity with the partial sequence of the *C. maenas* peptide. The O-glycosylated peptides from *D. melanogaster* (Bulet *et al.*, 1993) and *P. apterus* (Cociancich *et al.*, 1994b) each contain 3 PRP motifs,. However, these do

not overlap with more than one motif in the *C. maenas* peptide. Moreover, they, the bee apidaecins and most likely the metalnikowins do not act by formation of channels or pores in the bacterial membrane, unlike most known antibacterial peptides (Casteels & Tempst, 1994; Cociancich *et al.*, 1994b; Chernysh *et al.*, 1996). Bee abaecins and silk worm lebocins are active against both Gram positive and Gram negative bacteria (Casteels *et al.*, 1990, Hara & Yamakawa, 1995) but show no significant overlap with the 6.5 kDa peptide from *C. maenas*. *Drosophila* metchnikowins are only active gainst Gram positive bacteria (Levashina *et al.*, 1995). Thus, it would appear that there is little similarity between the *C. maenas* 6.5 kDa peptide and the proline rich antibacterial peptides already described from insects.

Several linear proline rich peptides are known from mammals (Gennaro *et al.*, 1989; Gudmundsson *et al.*, 1995; Harwig *et al.*, 1995) and the 6.5 kDa antibacterial peptide in *C. maenas* shares close sequence homology with the bovine proline and arginine rich peptide Bac 7. The largest shared sequence consists of the seven amino acids PRPLPFP, which form the beginning of a tetradecamer with three tandem repeats in Bac 7 (Frank *et al.*, 1990). Apart from these, the two sequences share the position of a proline, two further PRP motifs, the position of a glycine and one RP motif. From the partial sequence of the *C. maenas* 6.5 kDa peptide, it is not clear whether or not any repeats larger than the PRP triplets occur in this peptide.

Although there is little homology within the proline and arginine rich peptides of mammals, they are similar in amino acid composition and spectra of activity (Storici & Zanetti, 1993), and the relative amount and spacing of certain amino acids may indicate functional similarity. Computer modeling has shown that Bac 5 and Bac 7 can assume amphiphilic helical structures (Skerlavaj *et al.*, 1990), and both peptides rapidly penetrate the outer and inner membrane of Gram negative bacteria (Skerlavj *et al.*, 1990). From the similarity of the partial sequence of the *C.*

maenas 6.5 kDa peptide to bovine Bac 7, it is possible that the *C. maenas* peptide acts in a similar manner.

The mammalian proline and arginine rich peptides, including Bac 7, belong to the cathelicidins, a protein family which has been identified in myeloid cells (Zanetti *et al.*, 1995). These have a highly conserved N-terminal sequence of the pre-pro peptides (Zanetti *et al.*, 1995). Conversely, the antibacterial peptides themselves are situated at the C-terminal end of the pre-pro peptides; the sequences of which are highly variable, so there is little sequence homology between mature cathelicidins (Zanetti *et al.*, 1995). Instead, they fall into several structural groups: a) peptides with one disulphide bond; b) peptides with two disulphide bonds; c) mostly α-helical peptides and d) peptides rich in certain residues (Zanetti *et al.*, 1995), They thus comprise four of the five chemical groups of antibacterial peptides reviewed by Boman (1995). It therefore appears that a wide array of different mammalian antimicrobial peptides are evolutionary linked, but their relationships cannot be inferred from comparisions of the mature peptides (Zanetti *et al.*, 1995). Instead, the highly conserved pro-region of the cathelicidins may aid their proper positioning within cellular granules (Zanetti *et al.*, 1995).

To determine whether or not the *C. maenas* 6.5 kDa antibacterial peptide is related to Bac 7, it is necessary to investigate whether it shares a similar pre-pro sequence to the cathelicidins. Preliminary Northern analysis of total RNA (2 μg and 4 μg per lane) from*C. maenas* hemocytes (granulocyte proportion ca. 36 %) has been conducted in collaboration with Dr. J. Sommerville (University of St. Andrews) and Prof. M. Zanetti and Dr. M Scocchi (Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, Trieste, Italy). The method for RNA extraction and blotting is given in Appendix D. Preliminary analyses have not produced a positive result, but the presence of a precursor to the *C. maenas* 6.5 kDa peptide, with sequence similarity to the cathelicidin proregion, cannot be ruled out. Possibly, the

probe was too specific to target a putative precursor with lower homology than found in the cathelicidin proregion in mammals. Alternatively, precursor mRNA may not be present in fully differentiated *C. maenas* haemocytes.

In conclusion, *C. maenas* hemocyte contain a constitutive 6.5 kDa proline rich antibacterial peptide. This peptide is dissimilar to any antimicrobial peptide described so far from the arthropods, but shares sequence similarity with Bac 7 from bovine neutrophils. However, the evolutionary relationship of the *C. maenas* 6.5 kDa antibacterial peptide to members of the mammalian cathelicidins, to which bovine Bac 7 belongs, cannot be inferred from N-terminal amino acid sequencing. Work is currently underway to determine whether or not the *C. maenas* 6.5 kDa antibacterial peptide has a precursor with sequence similarity to the proregion of cathelicidins.

Table 3.3.1 Protein concentration and antibacterial activity against *P. immobilis* in pooled fractions of HLS pre-purified by SepPak C_{18} extraction and gel filtration

[1] Sample	[2] Fractions	[3]Volume (ml)	[4] Protein (mg ml^{-1})	[5] Inhibition zones (mm^2)
Pre-purified HLS (Sep-Pak C_{18})	[5] N/A	1.0	0.53 ± 0.04	29.77 ± 3.13
I	22-23	0.1	0.15 ± 0.03	0
II	25-32	0.4	1.30 ± 0.02	31.41 ± 2.42
III	37-40	0.2	0.05 ± 0.03	0
IV	42-43	0.1	0.04 ± 0.03	0

[1] The second fraction of HLS eluted with 20 % ACN and both fractions of HLS eluted with 40% ACN from a Sep Pak C_{18} cartridge (Sample) were pooled and subjected to gel filtration as described in Materials and Methods. The pools (I-IV) represent absorption peaks at 280 nm.

[2] Fraction size was 1 ml.

[3] After freeze-drying, pools were reconstituted 50 µl DW for each fraction included in them.

[4] Values represent means $\pm$ SE, n = 3.

[5] N/A = not applicable

Figure 3.3.1 Cation exchange chromatography of HLS pre-purified by gel filtration. Fractions from a Sephadex G-75 column were pooled, dialysed as described in Materials and Methods and applied to a 2 ml column of CM-Sephadex. Proteins were eluted and fractions collected as described in Materials and Methods. The fractions were assessed directly for activity against *P. immobilis* or *M. luteus*.

Figure 3.3.1

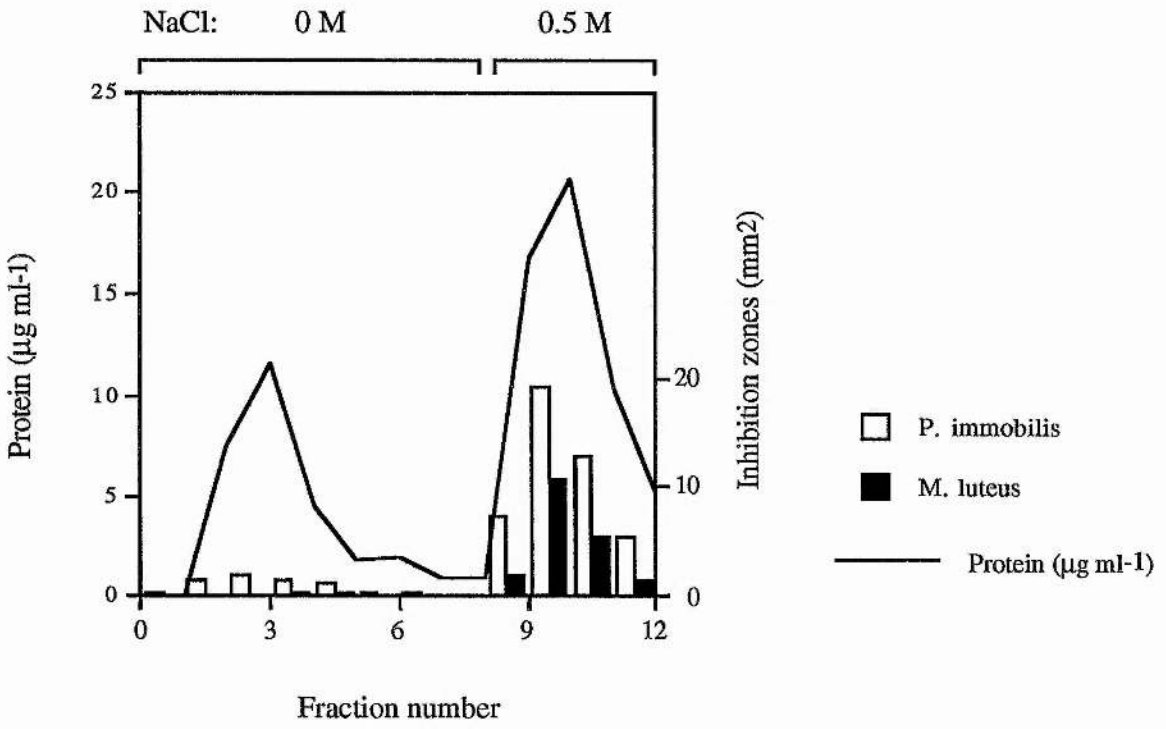

NaCl:
0 M
0.5 M
Protein (µg ml-1)
Inhibition zones (mm2)
Fraction number
P. immobilis
M. luteus
Protein (µg ml-1)

Figure 3.3.2 Reverse phase HPLC and antibacterial activity against *P. immobilis* or *M. luteus* of HLS. The HLS was pre-purified by SepPak C_{18} extraction, followed by gel filtration (Figure 3.3.2 a, b), or by gel filtration followed by cation exchange chromatography (Figure 3.3.2 c, d). The samples were prepared as described in Table 3.3.1 (Figure 3.3.2 a, b) or in the legend to Figure 3.3.1 (Figure 3.3.2 c, d). Trifluoroacetic acid and ACN were added to the samples to final concentrations of 0.1% and 10%, respectively, and samples were eluted with a 25-75% step gradient of ACN in 0.1 % TFA at a flow rate of 80 µl min^{-1}. Eighty microlitre fractions were collected and assayed for activity against *P. immobilis* or *M. luteus*. Figure 3.3.2 a) and c) show the absorbance profiles of the samples at 214 nm. Figure 3.3.2 b) and d) show the corresponding antibacterial activity against *P. immobilis* or *M. luteus*.

Figure 3.3.2

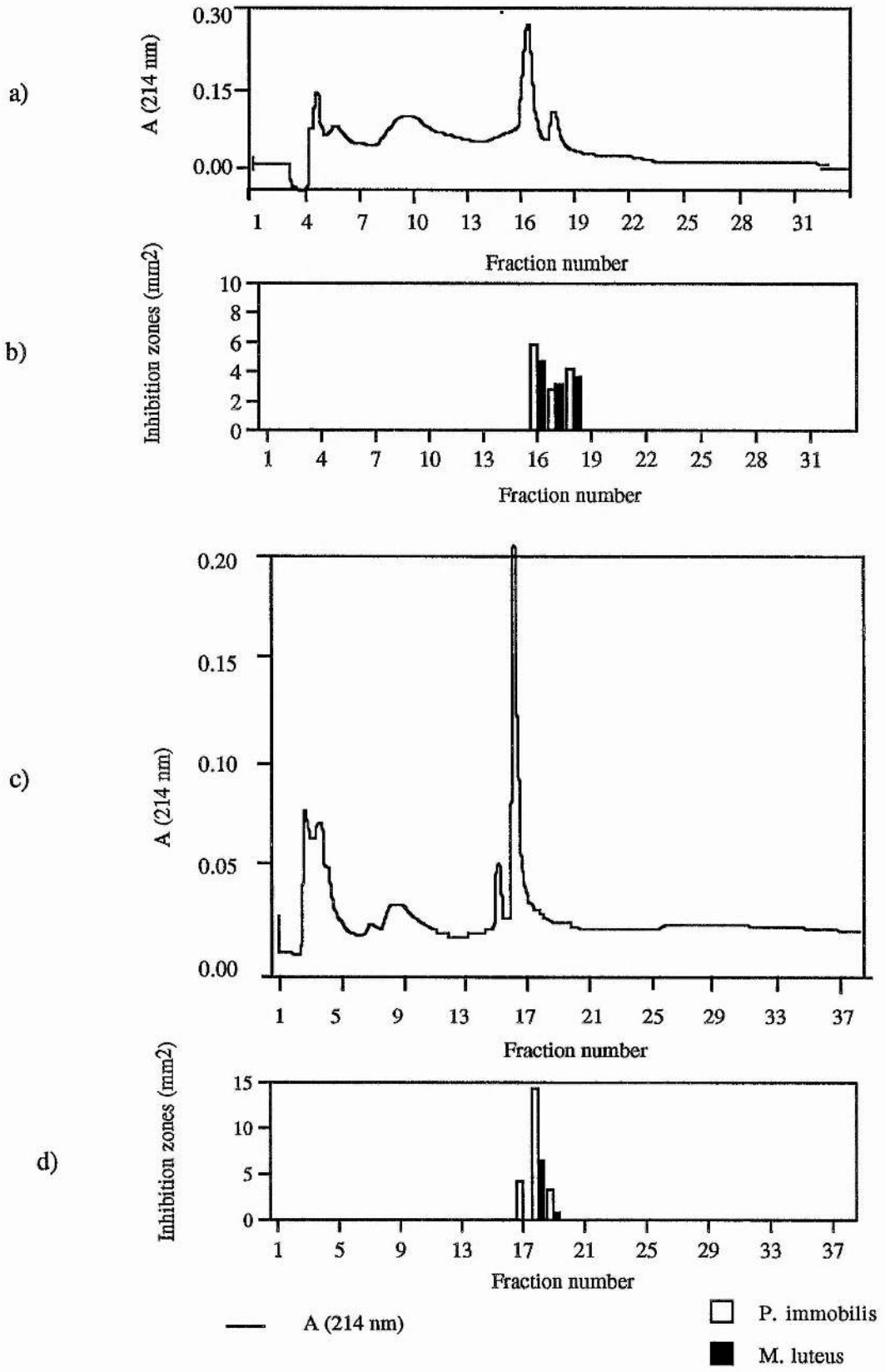

a)
A (214 nm)
0.30
0.15
0.00
1 4 7 10 13 16 19 22 25 28 31
Fraction number
b)
Inhibition zones (mm2)
10
8
6
4
2
0
1 4 7 10 13 16 19 22 25 28 31
Fraction number
c)
A (214 nm)
0.20
0.15
0.10
0.05
0.00
1 5 9 13 17 21 25 29 33 37
Fraction number
d)
Inhibition zones (mm2)
15
10
5
0
1 5 9 13 17 21 25 29 33 37
Fraction number
A (214 nm)
P. immobilis
M. luteus

Figure 3.3.3 Purification of a 6.5 kDa antibacterial peptide from *C. maenas* HLS. Haemocyte lysate supernatant was pre-purified as described in Materials and Methods. Fifty microlitres of the sample in DW containing 0.1% TFA was applied to a Nucleosil C_{18} column and eluted with a two-step linear gradient of 25-60% ACN. The flow rate was 1 ml min^{-1}. One millilitre fractions were collected, freeze-dried, recontituted in 100 µl DW, assayed for activity against *P. immobilis* or *M. luteus*, and subjected to SDS-PAGE. To 35 µl of fraction 53, 70 µl of 15% ACN, 0.1% TFA was added, the fraction was applied to a Micropore C_8 column and eluted as described in the legend to Figure 3.3.2.

The main graph shows the elution profile of pre-purified HLS from the C_{18} column at 224 nm and the ACN gradient. The histogram shows antibacterial activity against *P. immobilis* or *M. luteus*. Above the graph and histogram, SDS-PAGE profiles of fractions 49 and 53 are shown. Protein bands are indicated by horizontal arrows. The inset shows the elution profile of fraction 53 from the Micropore C_{18} column in the presence of the ACN gradient. Fraction 18 contained the purified peptide (inset).

Figure 3.3.3

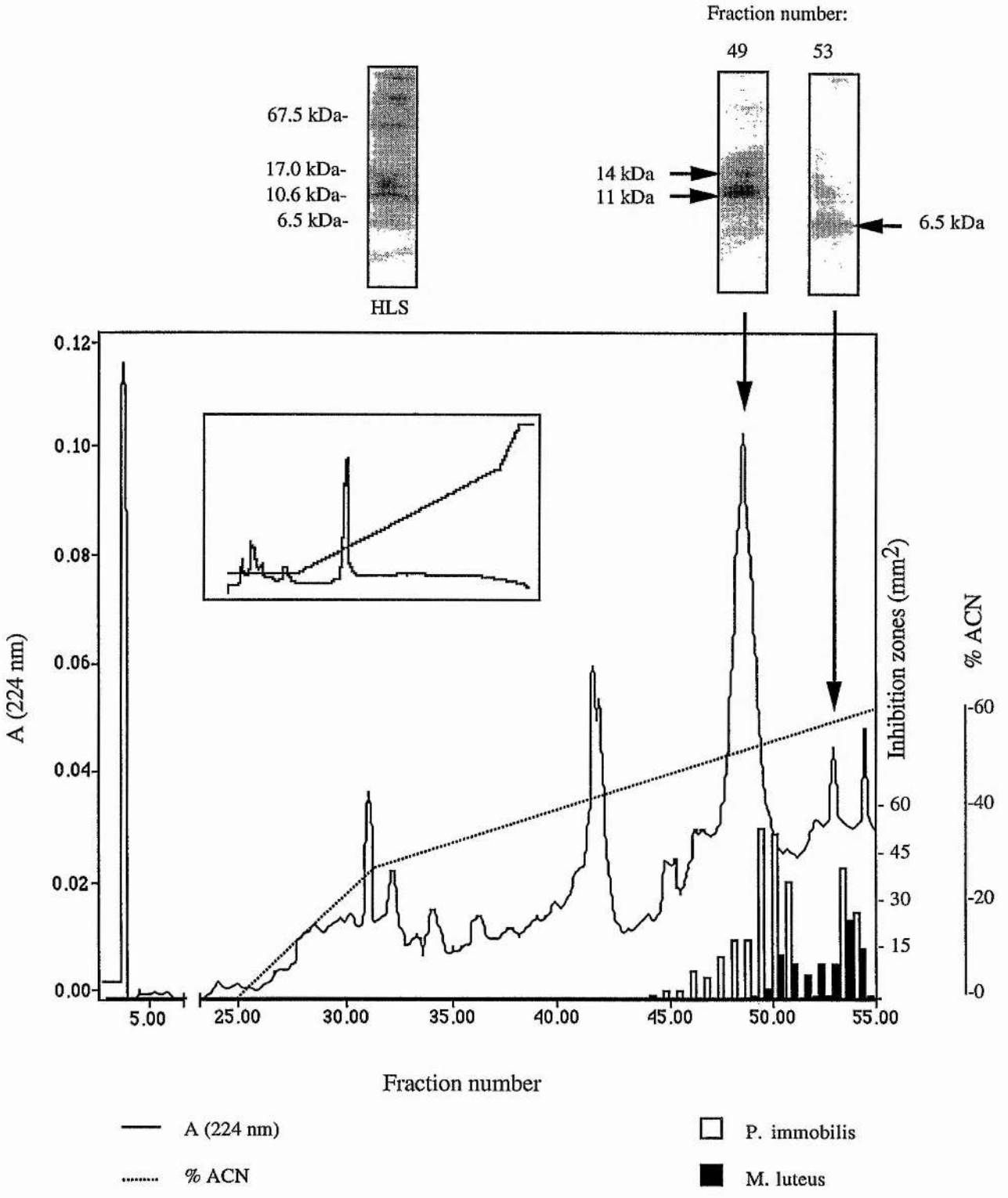

Fraction number:
49
53
67.5 kDa-
17.0 kDa-
10.6 kDa-
6.5 kDa-
HLS
14 kDa
11 kDa
6.5 kDa
A (224 nm)
Inhibition zones (mm2)
% ACN
Fraction number
A (224 nm)
% ACN
P. immobilis
M. luteus

Figure 3.3.4 Partial N-terminal amino acid sequence of the *C. maenas* 6.5 kDa antibacterial peptide and overlap with mature Bac 7 from Bac 7 residue 16. Twenty microlitres of Fraction 18, obtained from re-chromatography of a partially purified HLS fraction on a Microbore C_8 column (see Figure 3.3.2), was applied to an Applied Biosystems Procise automatic sequencer and subjected to automated Edman degradation. The sequence match was obtained with GCG FASTApep software, using the SwissProt database, version 29.0. This 28 amino acid overlap shows a homogeneity of 60.7% between the *C. maenas* 6.5 kDa peptide and bovine Bac 7. Identical residues are indicated in bold.

Figure 3.3.4

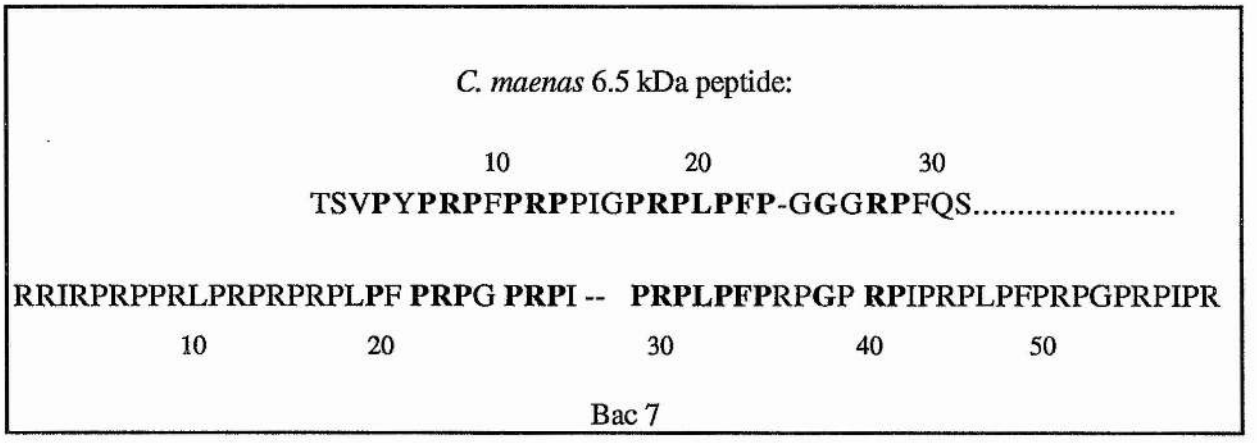
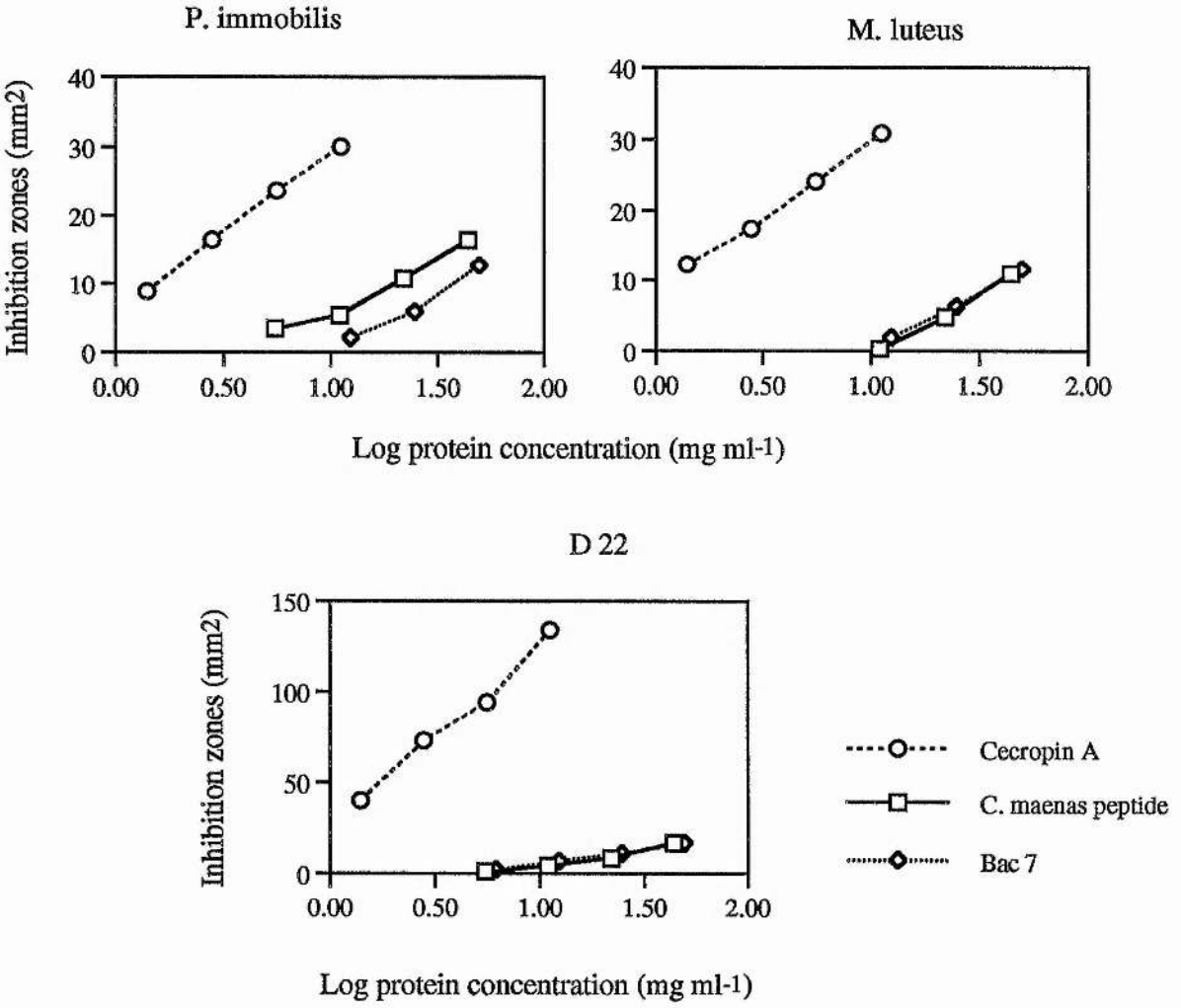

C. maenas 6.5 kDa peptide:
10
20
30
TSVPYPRPFPRPPIGPRPLPFP-GGGRPFQS.....
RRIRPRPPRLPRPRPRPLPF PRPG PRPI -- PRPLPFPRPGP RPIPRPLPFPRPGPRPIPR
10
20
30
40
50
Bac 7

Figure 3.3.5 Comparision of antibacterial activity of *C. maenas* 6.5 kDa antibacterial peptide to pure Cecropin A and Bac 7, using *P.immobilis*, *M. luteus* or *E. coli* D22 as test bacteria. Two-fold serial dilutions of each peptide were assayed by the radial diffusion assay as described in Materials and Methods. Assays of all three peptides were carried out on the same petri dish (one for each bacterium) to minimise variation in zone diameters due to variability in agarose thickness between plates.

Figure 3.3.5


P. immobilis
M. luteus
Inhibition zones (mm2)
40
30
20
10
0
0.00
0.50
1.00
1.50
2.00
Log protein concentration (mg ml-1)
D 22
150
100
50
0
Cecropin A
C. maenas peptide
Bac 7

3.4 INDUCTION OF ANTIBACTERIAL ACTIVITY IN THE PLASMA OF *C. MAENAS*

Introduction

So far, the chapters in this section have focussed on constitutive antibacterial factors found in the haemocytes of *C. maenas*. Previously, plasma of unchallenged *C. maenas* has been reported to be devoid of antibacterial activity (Chisholm & Smith, 1992). However, early studies on antibacterial activity in the plasma and serum of spiny lobsters, *Panulirus* spp.(Evans et al., 1968; 1969) and the American lobster, *Homarus americanus* (Acton et al., 1969; Stewart & Zwicker, 1972; Mori & Stewart, 1978), have claimed that inducible factors may be present in the haemolymph of Crustacea. The appearance of inducible antibacterial factors, following septic injury, is well documented in insects (Cociancich *et al.* 1994; Hoffmann, 1995), but the nature of inducible antibacterial factors in the Crustacea has not been investigated. Work was thus directed at investigating whether or not antibacterial proteins can be induced in the plasma of *C. maenas*, following a primary exposure to bacteria.

In *Panuris* spp. (Evans *et al.*; 1968, Evans *et al.*, 1969) and *H. americnus* (Stewart & Zwicker, 1972; Mori & Stewart, 1978) the antibacterial response in the plasma increases rapidly in the first two days following an injection of bacteria, and slowly declines over a period of several weeks. In the present chapter, antibacterial activity in *C. maenas* plasma was verified at 6 or 4 days after bacterial challenge, and the appearance of antibacterial factors in the plasma was monitored over 48 h post injection.

P. immobilis was chosen to challenge specimens of *C. maenas*. This bacterium is rapidly, but not completely, cleared from the circulation of *C. maenas* (Smith & Ratcliffe, 1980), and is highly susceptible to antibacterial factors in *C. maenas* HLS

(Chisholm & Smith, 1992). It has been shown to have the potential to grow well in cell-free *C. maenas* plasma (Chisholm & Smith, 1992).

Materials and Methods

Treatment of crabs

Specimens of *C. maenas* were collected and maintained as described in Chapter 2.1 (page 64). They were kept in the aquarium for no more than two weeks prior to use and only healthy male crabs with a carapace width of 7-8 cm were selected for experiments. Viable *P. immobilis* were prepared as described in Chapter 3.1 (page 130). Groups of 4-5 experimental animals were given an injection of ca. 1 x 10^7 bacteria, in 100 µl sterile CS, into the unsclerotized membrane at the base of the carapace and incubated at 10-12°C in a separate seawater container with air supply until use. Control animals were left untreated and similarly maintained.

Preparation of plasma

Plasma from experimental or control animals was prepared as described in Chapter 2.3 (page 111). Briefly, sterile syringes and needles were pre-chilled on ice. Crabs were bled from the unsclerotised part of a cheliped, using a 23 gauge needle, fitted to a 2 ml syringe. Half a millilitre of haemolymph was drawn into the syringe, transferred to pre-chilled tubes and centrifuged immediately at 600 *g* for 10 min to remove the haemocytes. Any samples showing foaming or haemocyte aggregation were discarded. After removal of haemocytes, plasma from control and experimental animals was pooled separately.

Antibacterial assays

Spread plate assays against *P. immobilis*, without addition of lysozyme, were carried out as described in Chapter 3.1 (page 130-132). Antibacterial activity was expressed as percentage of colony forming units (cfu) in the sample as compared to the control (CS). Radial diffusion assays against *P. immobilis*, *M. luteus*, or *E. coli* D22 were carried out as described in Chapter 3.1 (page 131-132), using PB as the underlay buffer.

Antibacterial overlays of native acid PAGE gels on *E. coli* D22, *P. immobilis* or *P. citreus* were prepared and stained as described in Chapter 3.2 (page 151).

Electrophoresis

SDS-PAGE under reducing conditions and native acid PAGE were carried out as described in Chapter 3.2 (page 147-148 and page 150, respectively). To avoid distortion of the gel by haemocyanin, dilute samples (corresponding to ca. 2 µl of plasma per lane) were applied. The gels were silver-stained as described in Chapter 3.3 (page 179).

Experiments

The presence of inducible antibacterial factors in *C.maenas* plasma was determined by giving five crabs injections of *P. immobilis* as described above and bleeding the animals at six days post infection. Five crabs, which had been left untreated, were also bled at this time. Plasma from treated or untreated crabs was pooled before assaying of antibacterial activity against *P. immobilis* by spread-plate assay.

The presence of antibacterial factors in *C. maenas* plasma was investigated by bleeding *C. maenas* (4 crabs per time-point) at 0 h, 0.25 h, 0.5 h, 1.5 h, 3.0 h, 9.0 h, 24 h and 48 h post injection with *P. immobilis*. Activity against *P. immobilis* in each sample was assessed by radial diffusion assay. An aliquot of each sample was also subjected to SDS-PAGE to ascertain whether or not new protein bands appear in the plasma. Plasma from crabs bled at 0 h, 24 h and 48 h was assessed for activity against *E. coli* D22 and used for antibacterial overlays on *E. coli* D 22, *P. citreus* or *P. immobilis*.

To assess variation between individual crabs as well as between treatment groups, plasma from four immunized and four untreated *C. maenas* was withdrawn at four days post injection and antibacterial activity against *P. immobilis* or *M. luteus* assessed by radial diffusion assay. Variation between individual crabs and between treatment groups was analysed simultaneously as described below.

Statistical analysis

The effect of bacterial injection on antibacterial activity in *C. menas* plasma was analysed by unpaired t-test as described by Campbell (1992).

Differences in antibacterial activity in the HLS or plasma of *C. maenas* at 0 h, 24 h or 48 h were analysed by one factor ANOVA as described by Zar (1984).

Differences in antibacterial activity between injected and untreated crabs (treatment groups) and between individual crabs (subgroups) were analysed simultaneously by nested ANOVA (Zar, 1984). To avoid the occurence of zero values where no inhibition zones were present, the measurements used for ANOVA analysis were total zone areas, including the area of the wells.

Results

The percentage survival of *P. immobilis* incubated in plasma of crabs which had received an injection of *P. immobilis* 6 days previously was 13.1 ± 1.4%, compared to 85.1 ± 3.9% for *P. immobilis* incubated in the plasma of untreated crabs (values represent means ± SE). This reduction of bacterial survival was highly significant ($P < 0.001$).

Figure 3.4.1 shows the induction of antibacterial activity against *P. immobilis* in the plasma of pre-challenged individual crabs over 48 h post injection. A rapid increase of antibacterial activity within 0.5 h post injection was followed by a slower increase of activity throughout the experimental period (Figure 3.4.1 a). However, variation between animals was high, as indicated by the standard errors (Figure 3.4.1 a). The SDS-PAGE profile of plasma from animals challenged at various time points indicated an increase of factors with molecular weights of < 14 kDa for samples obtained at > 9 h post injection (Figure 3.4.1 b). Two of these factors are indicated by arrows (Figure 3.4.1 b).

Table 3.4.1 shows antibacterial activity against *E. coli* D 22 in *C. maenas* HLS, or plasma at 0 h, 24 h or 48 h post injection. Antibacterial activity of the plasma increased with time after primary challenge, and the inhibition zones produced by plasma from crabs challenged at 48 h post injection were comparable to those of HLS (34.4 ± 2.8 mm^2 or 30.9 ± 0.9 mm^2, for plasma or HLS, respectively) (Table 3.4.1). The samples shown in Table 3.4.1 were used for native acid PAGE and overlays on *E. coli* D22 (Figure 3.4.2). Figure 3.4.2. a) shows a PAGE gel of HLS and plasma at 48 h, 24 h and 0 h post injection and the corresponding overlay on *E. coli* D22. Few bands are present on the PAGE gel for plasma samples at 0 h

and 24 h post injection, whereas several bands can be seen in the plasma sample at 48 h post injection (Figure 3.4.2 a). It is not apparent from this gel whether or not any of these proteins are different from proteins present in HLS (figure 3.4.2 a). The corresponding overlay shows three clear zones in the lane with whole HLS and a clear zone in the lane with plasma at 48 h post injection, but not at 24 h or 0 h post injection (Figure 3.4.2 a). The clear zones in overlays of HLS or of plasma at 48 h, 24 h or 0 h on *E. coli* D22 are shown more clearly in Figure 3.4.2 b. Overlays of plasma on *P. immobilis* or *P. citreus* produced no defined bands of activity.

Antibacterial activity against both *P. immobilis* and *M. luteus* is present in *C. maenas* plasma at four days post injection (Table 3.4.2). However, antibacterial activity was also present in one of the four untreated crabs (inhibition zones of 4.1 ± 0.3 mm^2 or 2.1 ± 0.8 mm^2 against *P. immobilis* or *M. luteus*, respectively) and the plasma of one of the treated crabs showed weak activity (inhibition zones of 2.0 ± 0.3 mm^2 or 1.5 ± 0.2 mm^2 against *P. immobilis* or *M. luteus*, respectively) (Table 3.4.2). For both bacterial strains, the difference between individual crabs was significant ($P < 0.001$ for *P. immobilis* and $P < 0.01$ for *M. luteus*). The difference between treatment groups (non-injected versus injected crabs) was non-significant ($P > 0.05$ for *P. immobilis* and $P > 0.5$ for *M. luteus*). However, for two treatment groups, the denominator of F has only 1 degree of freedom.

Discussion

Smith & Ratcliffe (1980a, 1980b) have previously shown that *C. maenas* is able to clear high doses of injected Gram negative and Gram positive bacteria from the haemolymph and that the bacteria are sequestered to the tissues (mainly the gills) by haemocyte aggregation. Work presented in this chapter shows that bacterial clearance may also be supplemented by the appearance of antibacterial factors in the

plasma of crabs within 0.5 h after injection with *P. immobilis*. This antibacterial activity persists for at least 6 days, although variation between individual crabs is high. For the American lobster, *Homarus americanus*, Stewart & Zwicker (1972) have shown that inducible antibacterial factors in the plasma require activation by haemocyte components. Antibacterial activity in the plasma of challenged animals was increased in the presence of haemocyte extracts from either challenged or control lobsters (Stewart & Zwicker, 1978). The authors concluded that the induced antibacterial activity in *H. americanus* plasma was a result of haemocyte rupture leading to the activation of antibacterial factors. The present work offers some support that antibacterial activity in the plasma of *C. maenas,* pre-challenged with bacteria, is probably due to haemocyte-derived factors. Thus, in overlays of PAGE gels on *E. coli* D22, a clear zone derived from plasma at 48 h post injection appeared at the same position as one of the clear zones derived from HLS. Furthermore, antibacterial activity increased very rapidly after primary bacterial exposure. This indicates that it is a result of haemocyte-derived factors rather than of *de novo* protein synthesis. In insects, genes controlling the synthesis of antibacterial proteins in the fat body or in certain haemocyte types are expressed within 30 min following immune challenge and continue transcription for 12-48 h (Hoffmann, 1995). However, a lag-phase is usually present before antibacterial activity appears in the plasma. In *Aedes aegypti* , activity appears at 2 h post injection (Chalk *et al.*, 1994), while in *Phormia terranovae* it appears at 3 h post injection (Cociancich *et al.*, 1994) and in *H. cecropia* and *Zophobas atratus*, it takes 5 h and 6 h, respectively (Hultmark *et al.*, 1980; Bulet *et al.*, 1991). In the case of *C. maenas*, antibacterial activity appears within 0.5 h post injection, without any such apparent lag-phase. Given the relatively slow metabolic rate of *C. maenas*, which was kept in seawater at 10-12 °C during these experiments, this rapid response supports the evidence that antibacterial factors in the plasma of pre-challenged crabs are probably hemocyte-derived.

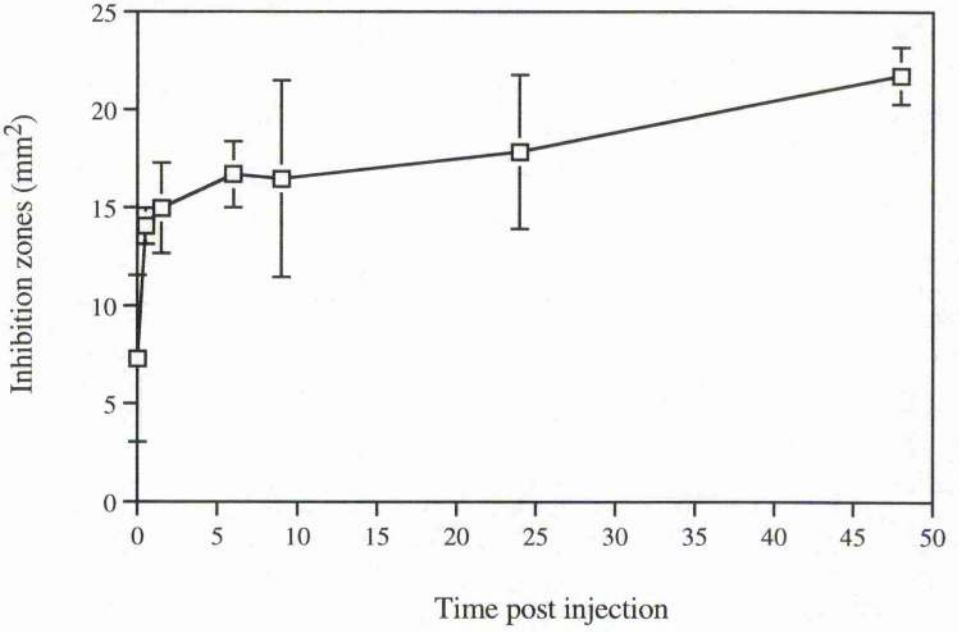
Antibacterial activity persists in the plasma of *C. maenas* for at least 6 days, and in the plasma of *Panuris* spp. (Evans *et al.*; 1968, Evans *et al.*, 1969) or *H. americanus* (Stewart & Zwicker, 1972; Mori & Stewart, 1978) for several weeks after primary bacterial challenge. This indicates that released antibacterial factors may have an important protective function against re-infection or re-growth of bacteria. Attempts to protect farmed penaeids by "vaccination" with killed *Vibrio* spp. cells, have produced some short-term increase in the growth of post-larvae (Giorgetti, 1990; Itami & Takakashi, 1991; Larramore, 1992) and production by broodstock (Giorgetti, 1990). Sung *et al.* (1995) recently showed that exposure of juvenile tiger shrimp to glucans in the water at 0.5-1.0 mg l^{-1} increased subsequent resistance to infection by *Vibrio* spp. and this effect lasted for 18 days. It is not known at this time whether or not continued release of antibacterial factors into the plasma takes place in the "vaccinated" animals or the factors are released just after exposure and are subsequently broken down gradually. To assess the value of such "vaccination" programs, it would therefore be interesting to study the synthesis and secretion of antibacterial factors in challenged crustaceans.

Figure 3.4.1. a) Antibacterial activity against *P. immobilis* in the plasma of *C. maenas* at various time points after challenge with ca. 1 x 10^8 bacteria. Injections were carried out and plasma was prepared as described in Materials and Methods. Antibacterial activity was determined as described in Chapter 3.1 (page 131-132). Values and error bars represent means and standard errors for three crabs.

b) SDS-PAGE analysis of plasma samples shown in Figure 3.4.1 a). Electrophoresis was carried out as described in Chapter 3.2 (page 147-148) and the gel was stained as described in Chapter 3.3 (page 179). Whole HLS and the position of molecular weight markers are shown on the right hand side of the gel. Arrows indicate bands which are more prominent in samples at 9-48 h post injection.

Figure 3.4.1

a)



b)

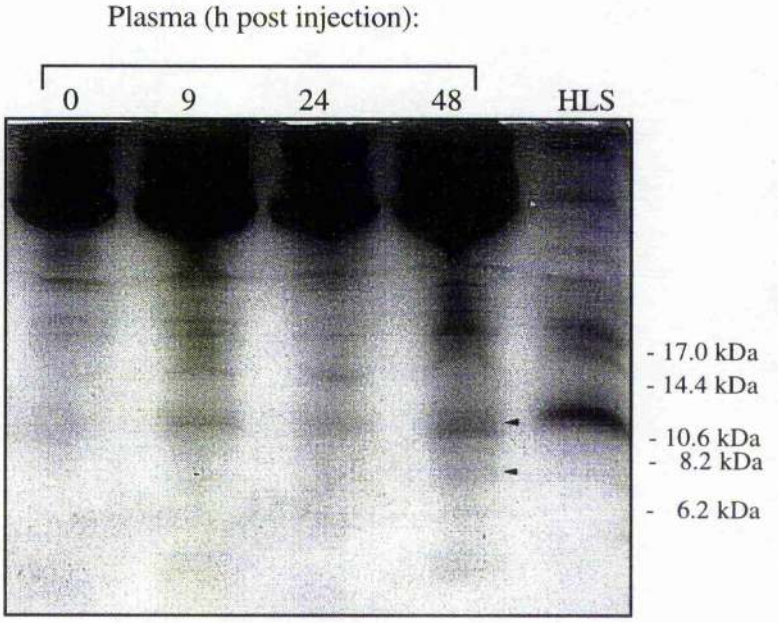


Table 3.4.1 Antibacterial activity against *E. coli* D22 in *C. maenas* HLS or plasma of *C. maenas* pre-challenged with *P. immobilis* .

Sample	[1] Time post injection (h)	[2] Activity against *E. coli* D22 (mm^2)
[3] Plasma	0	12.8 ± 0.5
Plasma	24	29.9 ± 3.6
Plasma	48	34.4 ± 2.8
[4] HLS (control)	[5] N/A	30.9 ± 0.9

[1] Crabs were given injections of 1 x 10^7 *P. immobilis* in 100 µl sterile CS

[2] Radial diffusion assays were carried out as described in Materials and Methods. Antibacterial activity is expressed as the area of the inhibition zone minus the area of the well. Values represent mean ± SE; n = 3.

[3] Plasma pooled from three crabs. Different crabs were used for each time post injection.

[4] HLS from 12 crabs, protein content of HLS ca. 4.3 mg ml^{-1}.

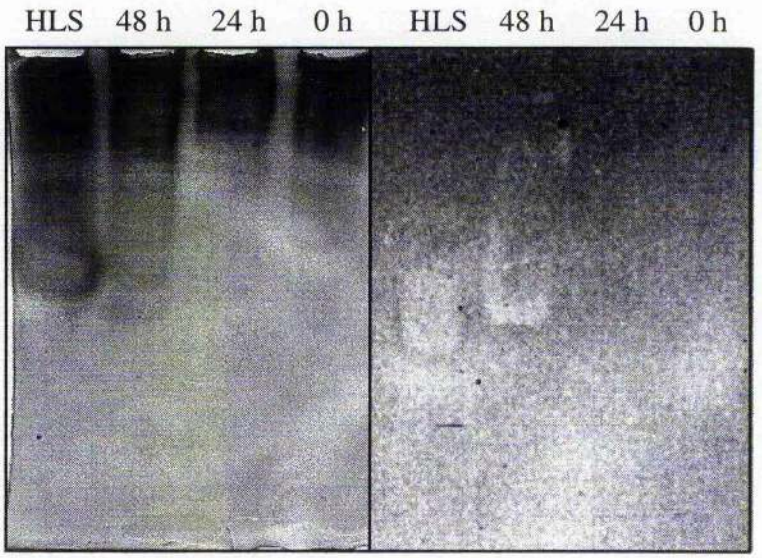
[5] N/A = not applicable.

Figure 3.4.2 a) Native acid PAGE of *C. maenas* HLS and plasma at 0 h, 24 h or 48 h post injection and overlay on *E. coli* D22. The PAGE gel and antibacterial overlay were prepared as described in Chapter 3.2 (page 149-150). The PAGE gel is shown on the left hand side of the figure and the overlay is shown on the right hand side. The arrow indicates a clear zone in the overlay of plasma at 48 h post injection.

b) Overlay of an acid native PAGE gel of *C. maenas* HLS or plasma at 48 h, 24 h or 0 h post injection on *E. coli* D 22, prepared as for Figure 3.4.2 a. The three arrows on the left hand side indicate clear zones of HLS, the arrow on the right hand side of the figure indicates the clear zone in the plasma samples at 48 h post injection.

Figure 3.4.2

a)



b)

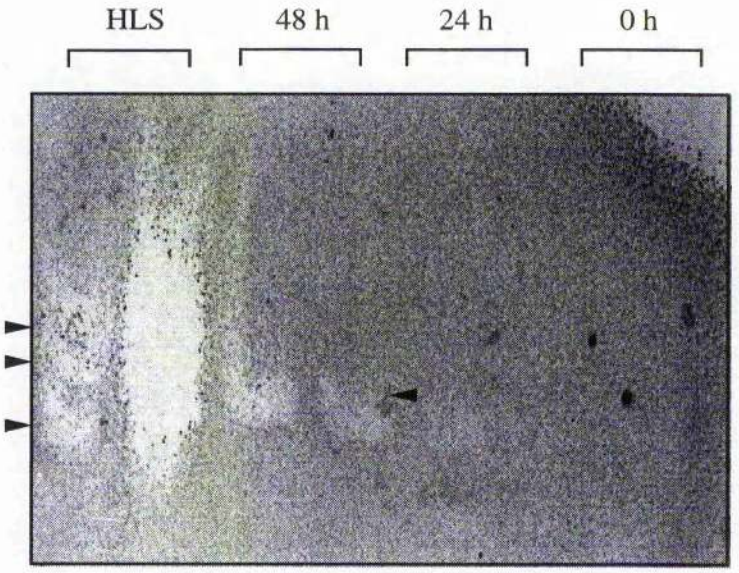


Table 3.4.2 Antibacterial activity against *P. immobilis* or *M. luteus* in the plasma of individual crabs left untreated or injected 4 days previously with *P. immobilis*

		[2] Inhibition zones (mm^2)	
[1] Treatment	Crab number	*P. immobilis*	*M. luteus*
	1	0.0	0.0
	2	0.0	0.0
Control	3	4.1 ± 0.3	2.1 ± 0.8
	4	0.0	0.0
	5	3.8 ± 0.6	3.8 + 0.6
	6	4.4 ± 0.7	3.6 ± 0.1
Injected	7	2.0 ± 0.3	1.5 ± 0.2
	8	8.6 ± 0.7	4.5 ± 0.4

[1] Experimental animals received an injection of ca. 1 x 10^7 viable *P. immobilis* four days prior to the experiment. Control animals were left untreated.

[2] Values are means ± SE for triplicate assays per individual crab.

4. GENERAL DISCUSSION

The first part of this thesis was aimed at detecting antiviral defence reactions in the shore crab, *C. maenas*. A starting point for this investigation was to determine whether or not *C. maenas* can recognize and remove viral particles *in vivo*. It was found that *C. maenas* is capable of clearing the coliphages T_2 and T_4, but not the marine bacteriophage Φ-111. The clearance curve for T_2 resembled that for the clearance of bacteria; a response which is associated with a decrease in haemocyte numbers (Smith & Ratcliffe, 1980b). However, a change in haemocyte counts was not observed in the present study. Given the small size of the phage particles and the low amount of material which comprises an injection dose of 10^8 plaque forming units (Garen & Kozloff, 1959), together with the high variability of haemocyte counts in *C. maenas* (Smith & Ratcliffe, 1980a), the lack of an obvious drop in the counts may not be surprising and is not indicative of a lack of haemocyte involvement.

To determine whether or not phage clearance is mediated by granular or semigranular haemocytes, activation of the proPO cascade by viruses was investigated *in vitro*. Activation of proPO by bacteriophages was not significantly higher than by the bacterial control extract, hence conclusions could not be drawn about the recognition of phages *in vitro*. However, it was found that the proPO cascade was activated by the *Chlorella* virus PBCV-1, a complex virus with several structural glycoproteins (VanEtten *et al.*, 1991). A high ratio of virus particles to protein was required (10^8 particles mg protein^{-1} ml^{-1}), but such a high concentration of viruses to effector molecules would be present if granular haemocytes aggregate and degranulate at sites of infection. Aggregations of haemocytes around virus infected tissues have been observed in crabs (Bang, 1974; Hoover & Bang, 1978) and shrimp (Nash *et al.*, 1988; Cesar *et al.*, 1992), although it remains unclear whether or not this response is directed against the infected cells or the viruses (Chapter 2.1, page 76).

Viruses lack peptidoglycans, LPS, or β-1,3 glucans, and if defence in crustaceans depends on the recognition of specific non-self, it is possible that some viruses are not recognized directly but instead, the host mounts a response towards infected cells or damaged tissues. In insects, some evidence for the role of infected haemocytes in antiviral defence *in vivo* has been obtained for the migratory grasshopper, *Melanoplus sanguinipes* (Miranpuri *et al.*, 1993). The haemocytes of *M. sanguinipes* show increased binding of lectins upon infection with an entomopoxvirus (MsEPV) (Miranpuri *et al.*, 1993). This may be indicative of an increase in cell membrane carbohydrates (Miranpuri *et al.*, 1993). Carbohydrates on haemocyte receptors have been linked to the formation of melatonic tumors in mutant *Drosophila* (Nappi & Silvers, 1984). Furthermore, Chen *et al.* (1995) have shown that lectins of the cockroach *Blaberus discoidalis* can activate the proPO cascade by binding to carbohydrate residues on haemocyte receptors. Using an infectious virus, it would be possible to determine whether or not changes in haemocyte membrane carbohydrates occur in response to viral infection in crustaceans *in vivo*.

Bang (1974) proposed that interferon-mediated defence might occur in virus-infected *C. maenas*. In vertebrates, interferons restrict viral replication when bound to glycoprotein receptors in the plasma membrane and internalized by the cell (Pestka *et al.*, 1987), but it is not known whether or not invertebrates synthesize interferons. Insect cell cultures infected with arboviruses, sometimes show an antiviral state, although "classical" interferon has not been detected (Riedel & Brown, 1979; Pudney *et al.*, 1982). Recently, it has been shown that the transcription of at least one of the *Drosophila* immune genes, diptericin, can be up-regulated by a factor binding to a sequence homologous to the one which binds interferon regulating factor IRF-1 in mammals (Georgel *et al.*, 1995). The presence of interferons in arthropods can therefore not be ruled out (Georgel *et al.*, 1995). It

might be possible to detect interferon genes in crustaceans by *in situ* hybridisation, provided that these molecules are similar to vertebrate interferons. As interferons are generally measured by their antiviral activity *in vitro* (Peska *et al.*, 1987), assessment of their importance in crustacean antiviral defence has to await the availability of suitable culture methods for crustacean cells.

An interesting model for the study of *in vivo* and *in vitro* antiviral defence in crustaceans against infectious viruses would be that of the two reoviruses W_2 of the mediterranean shore crab, *C. mediterraneus*, and P of the swimming crab, *Macropipus depurator* (Mari & Bonami, 1986). These two viruses differ little in size, density (Mari & Bonami, 1986), genome or protein composition (Montanie, 1992). Yet, W_2 is a specific for *C. mediterraneus* and does not infect *M. depurator* whereas P-virus infects *M. depurator* but not *C. mediterraneus* (Prof. J.R. Bonami, Laboratoire de Pathologie Compareé, Montpellier, France, pers. com.). It is possible that infectivity is due to differences between receptor molecules or defence responses in the two species of crabs. This combined system therefore provides a unique opportunity to investigate a highly specific infection-defence pathway in crustaceans (Bonami, pers. com.). The two viruses can be readily purified by solvent extraction and density-gradient centrifugation (Mari & Bonami, 1988), allowing sensitive assays *in vitro* as well as *in vivo*. *In vitro* assays could include respiratory burst assays (Bell & Smith, 1992), proPO activation assays and neutralisation assays with purified factors, such as the *C. maenas* 6.5 kDa peptide.

A shortcoming of the study of antiviral defence in crustaceans is the difficulty in propagating infective viruses *in vitro*, although some progress has been made with shrimp viruses (Chen & Kou, 1989; Lu *et al.*, 1995). One aim of this study was to attempt primary culture of *C. maenas* cells and some preliminary investigations have been carried out, although they have met with limited success. Hyaline cells were used as convenient models because clean preparations can easily

be obtained (Smith & Söderhäll, 1983). The cells remained viable for up to 4 days in the insect tissue culture medium TC-100, prepared and supplemented as described in Chapter 2.3 (page 112) with the addition of NaCl to a final concentration of 3.2%. However, viability declined from ca. 96% at 48 h to ca. 45% after 96 h (Appendix F). It was found that the pH of TC-100 (6.4) differed considerably from the physiological pH of *C. maenas* plasma (7.4) and pH alteration of TC-100 was impossible without precipitation of medium components. Precipitation of media components was also observed upon addition of Ca^{2+}, Mg^{2+}, and KCl to Glasgow Minimum Essential Medium, supplemented with NaCl as above, and upon pH adjustment of double strength Leibovitz's L-15 medium, modified according to Chen & Kou (1989) and supplemented with NaCl as above. Attempts at primary cultivation of *C. maenas* cells were subsequently discontinued, but work is currently under way in this laboratory to develop *in vitro* culture of tissues or haemocytes of several species of crabs. The possibility to detect the transcription of crustacean viruses with genomic probes (Bruce *et al.*, 1993; Mari *et al.*, 1993) after as little as 6 h post infection (Bonami, pers. com.) means that even short term primary cell culture can be of value for the study of antiviral defence.

The second part of this thesis was concerned with the nature of antimicrobial factors, which have been detected in *C. maenas* haemocytes (Chisholm & Smith, 1992) and which may have antiviral activity. This work followed from *in vitro* neutralisation assays against a range of bacteriophages or animal viruses with tissue- and haemolymph extracts of *C. maenas*. A weak non-specific neutralisation response against two viruses (AcNPV and parainfluenza) was detected in the digestive juice of *C. maenas*, but antiviral activity was not detected in the other extracts (Chapter 2.3, pages 116-117). However, *in vivo* neutralisation of viruses may require high local concentrations of antiviral factors, such as may be present within cellular granules (Daher *et al.*, 1986). Assays should therefore also be carried out with concentrated factors proposed to have antiviral activity.

C. maenas HLS is known to contain several antibacterial proteins, at least one of which was thought to be a peptide (Chisholm, 1993). Antimicrobial peptides have been identified as important components of invertebrate defence (Cociancich *et al.*, 1994, Hoffmann, 1995; Boman, 1995) as well as innate immunity of vertebrates (Barro & Simmacco, 1995; Boman, 1995; Ouellette & Selsted, 1995; Zanetti *et al.*, 1995). The potential applications for antimicrobial peptides have led to an increased effort to find new structures (Boman, 1995). Thus, the increased occurrence of antibiotic-resistant pathogenic bacteria (Swartz, 1994) has attracted interest in antimicrobial peptides as potential therapeutic agents (Boman, 1995). Certain peptides, such as frog skin magainins, are also specifically cytotoxic to certain tumor cells, and may be developed as anticancer drugs (Cruciani *et al.*, 1991). Most of the effort of identifying new antimicrobial peptides has focussed on insects (Hoffman, 1995; Cociancich, 1994), amphibians (Barra & Simmaco, 1995) or mammals (Selsted & Oullette, 1995; Boman, 1995; Zanetti *et al.*, 1995). In the present study, a 6.5 kDa peptide with sequence similarity to bovine Bac 7 was purified from *C. maenas* HLS (Chapter 3.3, page 187). This study is the first to describe an antibacterial peptide from a crustacean.

It was an unexpected finding that the *C. maenas* 6.5 kDa antibacterial peptide is more similar to a mammalian peptide, bovine Bac 7, than to peptides known from invertebrates. However, analogies between antimicrobial peptides of invertebrates and vertebrates have often been found. The best known are the defensins of insects and mammals, which share sequence homologies, although they differ in secondary structure (Cociancich *et al.*, 1994). Similarly, the cecropins of insects are similar to a cecropin from pig intestine (Lee *et al.*, 1989). Remarkably, bovine Bac 7 belongs to a family of myeloid proteins, the cathelicidins, which includes several antimicrobial peptides with highly conserved pro-regions but little sequence homology of the mature peptides (Zanetti *et al.*, 1995). Future work should be aimed at determining

the nature of the putative pre-pro-form of the *C. maenas* 6.5 kDa peptide to determine whether or not it is evolutionary related to cathelicidins.

Preliminary blotting of total haemocyte RNA from *C. maenas* with probes against the pro-region of Bac7 have not yielded any results (Dr. V.J. Smith & Prof. M. Zanetti, University of Trieste, Italy, pers. com.). To determine the size of the pre-pro peptide, blots of haemocyte extracts, prepared in the presence of protease inhibitor cocktails, can be probed with antibody to the mature peptide (Zanetti *et al.*, 1990). The pre-propeptide can be purified from these extracts by immuno-precipitation or affinity purification with the antibody (Zanetti *et al.*, 1990) and sequenced directly. However, a cheaper option would be to prepare a cDNA clone, either from granular and semigranular haemocyte mRNA or, if the peptide is not synthesized in the mature haemocytes, from haemopoietic tissue mRNA, and to determine the nucleotide sequence of this precursor.

From the sequence similarity of the *C. maenas* 6.5 kDa peptide to Bac 7, it may be anticipated that the mechanism of antibacterial activity of the *C. maenas* peptide resembles that of Bac 7 and Bac 5. These two bactenecins penetrate both the inner and the outer membrane of Gram negative bacteria and interfere with respiratory processes (Skerlavaj *et al.*, 1990). Bacterial membrane permeabilisation can be detected by the appearance of activity of intracellular β-galactosidase and, in the case of Gram negative bacteria, periplasmic β-lactamase in the medium (Skerlavaj *et al.*, 1990). The mechanism of antibacterial activity of the *C. maenas* 6.5 kDa peptide can thus be quickly assessed. Future work could also investigate a possible synergistic effect between the 6.5 kDa peptide and other components of *C. maenas* HLS. Indeed Skerlavaj *et al.* (1990) have reported a synergistic effect between Bac 7 or Bac 5 and lactoferrin from bovine neutrophil granules. Lactoferrin promotes LPS release from the outer membrane of Gram negative bacteria, thereby making it more accessible (Skerlavaj *et al.*, 1990). In the present study, it has been

observed that lysozyme acts synergistically with antibacterial factors in *C. maenas* HLS (Chapter 3.1, page 136) and this could be due to a similar increase of accessability by the 6.5 kDa peptide to the inner membrane of Gram negative bacteria. In *C. maenas*, lysozyme does not appear to act in antibacterial defence (Chisholm, 1993) but *C. maenas* haemocytes contain several antibacterial proteins in addition to the 6.5 kDa antibacterial peptide (Chapter 3.2), and it would be interesting to investigate whether or not these factors act synergistically.

The *C. maenas* 6.5 kDa antibacterial peptide may act in the long-term, as well as acutely, in response to bacterial infection. Antibacterial activity appears in *C. maenas* plasma within 30 min after injection of bacteria and persists for several days (Chapter 3.4, page 208-209). From the present study, it remained unclear which factors are responsible for this activity, but it is possible that the 6.5 kDa peptide is actively synthesized and secreted into the plasma of infected animals.

Linear proline rich antimicrobial peptides may have multiple physiological functions (Gallo *et al.*, 1994). The porcine proline-rich cathelicidin PR-39 is secreted into wound-fluid where it induces the formation of syndecans which signal cell responses in wound repair in addition to acting as an antimicrobial agent (Gallo *et al.*, 1994). It is possible that the *C. maenas* 6.5 kDa antibacterial peptide similarly signals cellular responses, and *in vitro* assays could be carried out with purified peptide to determine whether or not it triggers degranulation of granular or semigranular haemocytes or acts in chemotaxis.

Some antimicrobial peptides of invertebrates (Rinehart *et al.*, 1981; Kobayashi *et al.*, 1984; Azumi *et al.*, 1990; Morimoto *et al.*, 1991; Murakami *et al.*, 1991) or vertebrates (Ganz *et al.*, 1985, Daher *et al.*, 1986; Selsted & Harwig, 1987; Zerial *et al.*, 1987; Gennaro *et al.*, 1989) have antiviral activity *in vitro*. Bovine Bac 7 is known to have antiviral activity against HSV I (Gennaro *et al.*, 1989).

However, another peptide with a similar amino acid composition, bovine Bac 5, is devoid of antiviral activity (Gennaro *et al.*, 1989). There is a difference of ca. 30% between the partial sequence of the *C. maenas* 6.5 kDa peptide and Bac 7. Small differences between the structure or composition of peptides may be important for their antiviral activity (Selsted & Harwig, 1987). Thus, antiviral activity of human, rabbit or guinea-pig defensins against HSV-1 is determined by the position of just two arginine residues (Selsted & Harwig, 1987). Assays of antiviral activity with the purified *C. manas* 6.5 kDa peptide are therefore required before it can be concluded whether or not it has an antiviral effect. Unfortunately, insufficient purified peptide was available towards the end of this study to carry out titrations of antiviral activity.

Future studies of *in vitro* antiviral activity could investigate the role of antiviral factors in the interaction between viruses and host cells. Cells can be pre-incubated with the factors prior to viral infection to determine whether or not they inhibit virus adsorption to the cell (Daher *et al.*, 1986; Azumi *et al.*, 1990; Morimoto *et al.*, 1991). In previous studies, pre-incubation of host cells with human defensin HNP-1 did not prevent subsequent infection with HSV 1 (Daher *et al.*, 1986) and pre-incubation of cells with the tunicate peptide halocyclamine A did not prevent infection with the fish virus IPN (Azumi *et al.*, 1990), although these viruses were inactivated by these peptides in titer reduction assays (Daher *et al.*, 1986) or neutralisation assays (Azumi *et al.*, 1990). However, pre-incubation of host cells with horseshoe crab tachyplesin may have protected host cells from infection with HIV (Morimoto *et al.*, 1991).

Overall, it appears from the present work that the antibacterial defence in *C. maenas* is more pronounced than its non-specific antiviral defence. One explanation for the presence of a strong antibacterial defence is that animals are exposed to many potentially infectious bacteria in the environment and antibacterial factors can therefore be expected to be widespread (Boman, 1995). Accordingly,

many species of free-living marine bacteria can act as facultative pathogens of crustaceans (e.g.*Vibrio* spp., *Aeromonas* spp., *Spirillum* spp.) (Stewart, 1993). In insects, the use of non-pathogenic bacteria for the study of insect immunity has greatly facilitated the discovery of immune proteins, to which pathogenic bacteria are largely resistant (Andersons *et al.*, 1990). However, it transpired that in the absence of these immune proteins, injected "harmless" bacteria kill the insects within 24 h (Boman, 1995). It therefore appears that there is no such thing as a "non-pathogenic" bacterium and this underlines the need for an efficient antibacterial defence in animals. This anticipatory response is facilitated by the fact that bacteria share common surface antigens, such as peptidoglycans or lipopolysaccharides, which can be targeted by semi-specific recognition pathways, such as the proPO cascade. This semi-specific response is efficient and does not require the production of large numbers of specific effectors (Boman, 1995). By contrast, viruses differ widely in structure and composition. Furthermore, viruses are numerous in the sea (Suttle & Chen, 1992), but most are non-infectious to crustaceans. There is therefore little possibility or requirement for crustaceans to evolve effector molecules against many different types of viruses. Instead, it is possible that antiviral defence is targeted at damage to infected tissues (wound response) or mediated by signalling factors from infected cells. Alternatively, because a virus has to enter a cell in order to replicate, intracellular neutralising factors may act at high local concentrations. Further work is therefore needed to resolve the nature of antiviral defence in crustaceans. The development of crustacean cell lines will greatly facilitate the titration of infectious crustacean viruses and the investigation of cellular defence responses *in vitro*.

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APPENDICES

Appendix A: Buffer Formulations

Blocking buffer for probing blotted proteins with HLS

(Aspán & Söderhäll, 1991)

Tris	0.05 M
Nacl	0.45 M
EDTA	5 mM
Triton-X 100	0.05 %
gelatin	0.25 %
pH	8.0

Sodium cocodylate buffer I (CAC I)

(Söderhäll *et al.*, 1986)

Sodium cacodylate	0.01 M
NaCl	0.45 M
pH	7.0

Sodium cocodylate buffer II (CAC II)

(Söderhäll *et al.*, 1986)

Sodium cacodylate	0.01 M
NaCl	0.45 M
pH	7.0

Sodium cocodylate buffer III (CAC III)

(Söderhäll *et al.*, 1986)

Sodium cacodylate	0.01 M
NaCl	0.45 M
$CaCl_2.6H_2O$	0.01 M
pH	7.5

Carcinus saline (CS)

(modified from Smith & Ratcliffe, 1978)

NaCl	0.45 M
KCl	13 mM
$CaCl_2 \cdot 6\ H_2O$,	30 mM
$MgCl_2 \cdot 6H_2O$,	0.26 M
Tris	0.05 M
pH	7.4

Marine anticoagulant (MA)

(Söderhäll & Smith, 1981)

NaCl	0.45 M
glucose	0.10 M
trisodium citrate	30 mM
citric acid	26 mM
EDTA	10 mM
pH	4.6

Marine salt magnesium buffer (MSM)

NaCl	0.45 M
$MgSO_4 \cdot 7H_2O$	12 mM
Tris,	0.05 M
gelatin	0.01 %
pH	7.5

Phosphate buffer (PB)

KH_2PO_4	1.5 mM
Na_2HPO_4	6.5 mM
pH	7.2

Salt magnesium buffer (SM)

(Maniatis *et al.*, 1982)

NaCl	0.1 M
$MgSO_4 \cdot 7\ H_2O$	12 mM
Tris	0.05 M
gelatin	0.01 %
pH	7.5

Towbin buffer (for Western blotting)

(Towbin, 1971)

Tris	25 mM
glycine	192 mM
methanol	20 %

Appendix B: Electrophoresis

SDS-PAGE

(modified from Schägger & von Jagow, 1987)

The quantities have been adapted for use with minigels (1 mm thickness).

Tris-HCl (pH 6.8)	Trizma base (Sigma)	1.21 g
(use freshly)	DW	7.0 ml
	HCl (conc)	to pH 6.8
	DW	to 10 ml

Sample buffer:	20 % SDS (w/v)	4.0 ml
	Glycerol	2.4 ml
	2-mercaptoethanol	0.4 ml
	0.05% Bromophenol blue (w/v)	200 µl
	Tris-HCl (pH 6.8)	1.0 ml
	DW	2.0 ml

Handle 2-mercaptoethanol in a fume hood. Best kept in aliquots at -20°C, but stable at room temperature for several weeks. This is a double-strength formulation, add equal volume of sample or DW if applied to freeze-dried samples. Heat samples at 60°C for 15 min and allow to cool to room temperature prior to run. Remaining samples can be kept in sample buffer at -20°C for future use.

Anode buffer (reservoir buffer)	Trizma base (Sigma)	7.26 g
(use freshly)	DW	50 ml
	HCl (conc)	to pH 8.9
	DW	to 300 ml

Cathode buffer	Trizma base (Sigma)	2.42 g
(use freshly)	Tricine	3.59 g
	SDS	0.2 g
	HCl (conc)	to pH 8.2
	DW	to 200 ml

Acrylamide-Bis	Acrylamide	24 g
(449.5 % T; 3 % C)	Bis-acrylamide	0.75 g
	DW	to 50 ml

Keeps at least 1 month at 4°C in dark bottle. The acrylamide may crystallise out at 4°C and may require agitation at room temperature before use. Crystalline acrylamide and bis-acrylamide are neurotoxins which are absorbed through the skin and act accumulatively. Take care to remove all spillage after weighing the ingredients.

Gel buffer	Trizma base (Sigma)	18.17 g
	SDS	0.15 g
	DW	ca 10 ml
	HCl (conc)	to pH 8.45
	DW	to 50 ml

Gentle warming and agitation is required to dissolve the Trizma. Keeps ca. 2 weeks at 4°C but the SDS may crystallise, bring to room temperature before use.

Ammonium persulphate (APS)	10 % w/v in DW. Prepare each day.

Gel composition:

	stacking gel	spacer gel	separating gel
Acrylamide-bis	0.50 ml	1.525 ml	3.33 ml
Gel buffer	1.55 ml	2.50 ml	3.33 ml
DW	4.20 ml	3.50 ml	2.27 ml
Glycerol	-	-	1.07 ml
TEMED	7.5 µl	3 µl	5 µl
APS	75 µl	30 µl	50 µl
Height	ca. 1 cm	ca. 1.5 cm	ca. 5.5 cm

Pour within 5 minutes of adding TEMED and APS . Run at constant current: 35 mA until the sample has entered the stacking gel, then 50 mA until the dye front has reached the bottom of the gel (ca. 1.5 h).

Native acid PAGE

(modified from Lehrer *et al.*, 1991).

Amounts are sufficient for two minigels (1 mm thickness). These gels are used for bacterial overlays as described in Chapter 3.2, page 161. Note that molecular weight markers cannot be used for native PAGE as separation depends on both size and charge of the sample proteins.

Sample buffer

Urea (electrophosis grade)	3.78 g (6 M)
Acetic acid (glacial)	1.0 ml
Methyl green	0.1 g
DW	to 10 ml

Double-strength formulation: add equal volume of sample, or DW if sample is freeze-dried. Keeps for several weeks at 4°C.

Acrylamide-Bis	Acrylamide	30 g
(61.6 % T,; 2.6 % C)	Bis-acrylamide	0.7 g
	DW	to 50 ml

Keeps at least 1 month at 4°C in dark bottle. The acrylamide may crystallise out at 4°C and may require agitation at room temperature before use. Crystalline acrylamide and bis-acrylamide are neurotoxins which are absorbed through the skin and act accumulatively. Take care to remove all spillage after weighing the ingredients.

Ammonium persulphate (APS): 10 % w/v in DW, use freshly.

Acetic acid-TEMED stock	Acetic acid (glacial)	1.30 ml
(use fresh)	TEMED	120 µl
	DW	1.58 ml

Running buffer: 5 % w/v Acetic acid (glacial) in DW.

Gel-composition	Urea	6.40 g
(pour within 5 min)	DW	9.0 ml
	APS	0.4 ml
	Acetic acid-TEMED stock	2.67 ml
	Acrylamide-bis	4.45 ml

Gels are run at reversed polarity (i.e. towards the cathode). Pre-run gels for ca. 45 mins at 200 V to remove excess APS and TEMED. Sample buffer can be loaded to check pre-run time. Completion of the run depends on the charge and size of sample proteins, for HLS the run is completed after the green component of the tracker dye has left the bottom of the gel.

Coomassie blue staining

Stain:	Coomassie brilliant blue	0.1 g
	Acetic acid (glacial)	10 ml
	Methanol	45 ml
	DW	45 ml

Keeps in a dark bottle at room temperature. Fix and stain gel simultaneously for at least 2 h or overnight.

De-Stain 10 % (w/v) acetic acid (glacial) in DW

Several changes every 30 min or so until background is clear.

Reversible copper staining

(Lee, C., Lewin, A. & Branton, D. (1987). Copper staining: A five-minute protein stain for sodium dodecyl sulphate polyacrylamide gels. *An. Biochem.* 166, 308-312.

With this method, proteins appear as clear bands against a whitish-blue background on SDS-gels. The method is 3 x as sensitive as Coomassie blue stain for SDS-gels, but not as sensitive for native gels, which have to be soaked in Tris-SDS prior to staining. After de-stain, the excised bands can be used for 2-D PAGE etc.

Stain 100 ml $CuCl_2$ per gel

Dip gel into DW foor 30-60 s then quickly immerse in stain. Rock for 5 min. wash with DW for 2-3 min and store under DW (do not dry).De-stain 3 x 15 min.

De-stain 0.25 M EDTA
0.05 M Tris
Conc HCl to pH 9.0

Silver stain

(BioRad silver stain kit rapid protocol for mini gels)

Fixative	40% (w/v) methanol, 10% (w/v) acetic acid (glacial) in DW
Developer	32 g l^{-1}, keep at 23-25°C for up to 1 month.
Oxidiser solution	10% w/v in DW. 100 ml is sufficient for 2 mini gels.
Silver stain solution	10% w/v in DW. 100 ml is sufficient for 2 mini gels.
DW for washing	at least 1 l.
Stop solution	5 % w/v) acetic acid (glacial) in DW

The staining is best carried out on a rocker or orbital shaker.

1. Fix gels 30 min. or overnight,

2. Completely immerse in oxidizer for 5 min,

3. Wash repeatedly in DW until all the yellow colour has disappeared. Wash for 15 min maximum, using 6 or more changes of water, especially during the first 5 min,

4. Completely immerse in silver reagent for 20 min,

5. Wash with DW, 30 s,

6. Immerse in developer 30 s or until brown precipitate appears,

7. Renew developer. If more precipitate appears pour off and add fresh developer,

8. Change developer every 5 minutes until just before contrast is satisfactory,

9. Immerse in stop solution.

Gels can be kept in DW almost indefinitely, although Coomassie blue bands will eventually fade. Gels sealed in polythene freezer bags can be scanned directly and analyzed with NIH image.

Appendix C: Purification of 6.5 kDa antibacterial peptide from *C. maenas* HLS

Schedule A: SepPak C_{18} extraction, gel filtration and RP-HPLC

1. Collect haemolymph (ca. 2.5 ml $crab^{-1}$) into an equal volume of ice-cold, sterile MA (Appendix A),

2. Centrifuge at 2000 *g* for 10 min at 4°C,

3. Wash haemocytes once in sterile, ice cold CS or CAC I (Appendix A), re-suspend in DW with 0.1% PTU (w/v),

4. Homogenize for 5 min on ice with a pre-chilled, sterile glass-piston homogenizer,

5. Clarify the homogenate by centrifugation at 48, 000 *g* for 20 min at 4°C,

6. Acidify by adding 1/10 of 1% TFA,

7. Wash SepPak C_{18} cartridge with 4 ml methanol and 4 ml 0.1 % TFA in DW, always keeping flow rates at max. 2 ml min^{-1},

8. Add up to 2 ml HLS,

9. Wash with 5 ml 0.1 % TFA in DW,

10. Elute with 3 ml 60 % ACN in 0.1 % TFA,

11. Freeze-dry and re-suspend in 0.5 - 1.0 ml of 0.1 M ammonium acetate, pH 6.5,

12. Apply to a Sephadex-G75 column with dimensions at least 0.9 x 35 cm, pre-equilibrated with 0.1 M ammonium acetate, pH 6.5,

13. Elute at a flow rate of 6 ml h^{-1}. This step is necessary to remove remaining PTU which can interfere with reverse phase HPLC. PTU will cause an absorbance peak just after V_t of the column, the column dimensions used assure separation of PTU from the preceeding peptide peak,

14. Pool the absorbance peaks at 280 nm, freeze-dry and assess antibacterial activity,

15. Re-suspend the active peak in up to 0.5 ml 0.1% TFA, 10 % ACN in DW,

16; Apply to a C_8 or C_{16} RP-HPLC column pre-equilibrated in 0.1% TFA in DW. Recommended column dimensions: 5 µm particle size, 300 Å pore size, 250 mm length, 5 mm inner diameter. Gradient on micropore system (flow rate 80 µl min^{-1}): 25% to 75% after 10 min wash, run over ca. 30 min. At a flow rate of 1 ml min^{-1}: 30% to 60% over at least 60 min. The ideal shape and steepness of the gradient and the flowrate (which can lead to baseline shift) must be determined experimentally for HPLCs other than the micropore system as the HPLC run shown in Figure 3.3.3 (page 208) did not give complete resolution.

Schedule B: Gel filtration, cation exchange chromatography and RP-HPLC

The ion exchange resin has been calibrated for separation of the peptide (modified from Roe, 1992) and calibration must be repeated for different proteins or peptides to optimise separation. The first purification step, gel filtration, is not necessarily required but samples pre-purified by ion exchange chromatography alone caused some clogging of the HPLC column.

Repeat steps 1-5;

6. Apply up to 1.0 ml to a Sephadex-G75 column with dimensions at least 0.9 x 35 cm, pre-equilibrated with 0.1 M sterile ammonium acetate, pH 6.5,

7. Elute at a flow rate of 6 ml h^{-1}. Collect the eluent during the middle third of the run, corresponding to fractions 42-57 in Figure 3.2.2 (page 178. Note this figure represents elution profile from a longer column). Alternatively, collect 1 ml fractions and pool accordingly after determination of antibacterial activity,

8. Freeze-dry and resuspend in sterile 0.05 M glycine, pH 8.5;

9. Wash a column of CM-Sephadex (volume 2-3 ml) with 5-10 bed volumes of sterile 0.05 M glycine, pH 8.5,

10. Apply the sample (volume is not particularly important, the column should bind several mg of protein),

11. Wash with 2-3 bed volumes of sterile 0.05 M glycine, pH 8.5,

12. Elute proteins of interest with 2 bed volumes of sterile 0.05 M glycine, 0.45 M NaCl, pH 8.5. Protein content and antibacterial activity can be determined directly as the buffer does not interfer with either assay. The sample will keep at 4°C overnight,

13. The best way to remove NaCl and glycine and concentrate the sample at the same time is ultrafiltration e.g. on an ultrafree-CL filter unit (Waters) with NMWL of 5 kDa. Alternatively, the sample can be dialyzed against DW, using dialysis membranes with NMWL $\leq$ 5 kDa and subsequently concentrated by freeze-drying,

14. Proceed with HPLC as above.

Two active peaks eluted from the HPLC column when purification scheme A was used. The first of these two contained two protein bands of ca. 11 and ca. 14 kDa. Samples prepared by Schedule B appeared to lack the 14 kDa component and showed one activity peak corresponding to the 6.5 kDa peptide.

Appendix D: Extraction of total RNA from *C. maenas* haemocytes for Northern blotting

This protocol is based on Maniatis *et al.* (1982) and modified by Dr. John Sommerville, University of St. Andrews.

Chemicals should be molecular biology grade and should be reserved for RNAse-free work. Glassware and spatulas for weighing chemicals should be baked at 180°C overnight. Alternatively, they can be treated with diethyl pyrocarbonate (DEPC), together with the water and buffers (except tris buffers): Add 0.1% w/v DEPC to the solutions and incubate overnight at 37°C. This will inactivate most RNAses. DEPC is removed by autoclaving for 15 min at 121°C.

RNA extraction

Lysis buffer	Guanidine HCl	8.0 M
	Sodium acetate	0.1 M
	Sodium lauryl sarcosinate	0.5 %
	β-2, mercaptoethanol	1.0 %

Add 19.1 g of guanidine HCl to 0.83 ml sodium acetate (pH 5.2) and 23.8 ml DW and mix, then add 1.25 ml 10% sodium lauryl sarcosinate. This solution is stable in a dark bottle at room temperature for several weeks. Just prior to use, add 1 µl β-2, mercaptoethanol per 100 µl lysis buffer.

Proteinase digestion buffer:	Tris	0.2 M
	EDTA	25 mM
	NaCl	0.3 M
	SDS	2 %

Adjust pH of Tris and EDTA to 8.0.

1. Collect haemolymph (ca. 2.5 ml crab $^{-1}$)into an equal volume of ice-cold, sterile MA (Appendix A),

2. Centrifuge at 2000 *g* for 10 min at 4°C,

3. Wash haemocytes once in sterile, ice cold 0.45 M NaCl, 0.05 M tris (pH 7.2),

4. Raise the pellet in 2 volumes lysis buffer, vortexing occasionally. Centrifuge 10, 000 *g* 10 min if necessary and keep supernatant,

5. Warm to 60°C, shear by drawing through a needle (23 G) into a syringe. Repeat until viscosity has decreased,

6. Add an equal volume of phenol, warm to 60°C, shear again,

7. Split into several tubes if necessary an add an equal volume of chloroform (containing 1:24 isoamyl alcohol),

8. Warm to 60°C, vortex until well mixed,

9. Centrifuge in microfuge at high speed for min and recover aqueous (upper) phase,

10. Re-extract with an equal volume of a 50/50 mix of phenol-chloroform,

11. Re-extract with chloroform (room temperature);

12. Collect the supernatants into fresh tube(s) and add an two volumes of ice-cold ethanol, store at -20°C overnight,

13. Centrifuge 10, 000 *g* 15 min 4°C. Rinse pellets with 70% ethanol. Dry (speedvac if available),

14. Raise pellets in a total of 50 µl DEPC treated DW and add 150 µl proteinase digestion buffer, 2 µl proteinase K (20 mg ml^{-1}),

15. Incubate 1 h 37°C,

16. Extract with an equal volume of phenol/chloroform,

17. Re-extract with an equal volume of chloroform,

18. Precipitate with 2 volumes of ethanol, keep at -20°C for at least 2 h, centrifuge as in step 13,

19. Raise pellet in 100 µl DEPC treated DW, add 100 µl 8 M LiCl and store at -20°C overnight,

20. Centrifuge at 10, 000 *g* 20 min at 4°C, rinse pellet once in 70% ethanol,

21. Raise in 10 µl DEPC treated DW or deionized formamide (for long term storage).

Electrophoresis (formaldehyde-agarose gel)

Wash electrophoresis tank with detergent, rinse with water, dry with ethanol, fill with 3% H_2O_2, leave 10 min and rinse with DEPC-treated water.

10 x gel running buffer:	MOPS (pH 7.0)	0.2 M
	Sodium acetate	80 mM
	EDTA (pH 8.0)	10 mM

Dissolve 41.2 g of MOPS (3-(N-morpholino)propanesulfonic acid) in 800 ml of DEPC treated 100 mM sodium acetate. Adjust pH to 7.0 with 2 N NaOH. Add 10 ml of DEPC treated 1.0 M EDTA (pH 8.0). Adjust to 1 l with DEPC treated DW. Filter-sterilize through 0.2 µm membrane. Store in dark bottle at room temperature. Stable for a while, discolours with time. Straw-coloured buffer is still OK but darker buffer is not.

Gel composition:	Agarose	1g
	DW	75 ml

Melt and keep in waterbath, then add:

10 x running buffer	10 ml
Formaldehyde	17 ml

If possible, pour in fumehood. Adjust volume for minigels. Formaldehyde refers to a 37% (12.3 M) solution. Check that pH of formaldehyde is > 4.0.

Samples:	RNA (up to 30 µg)	4.5 µl
	10 x running buffer	1.0 µl
	*DW (DEPC-treated)	1.0 µl
	Formaldehyde	3.5 µl
	Formamide (deionized)	10 µl

* If pre-staining is desired, this would instead consist of 1 µl of ethidium bromide (1 mg ml^{-1}), but this can reduce the efficiency of the Northern transfer.

Incubate at 65°C for 15 min and chill on ice, centrifuge 5 s to collect fluid, add 2 µl DEPC-treated gel loading buffer:

Gel loading buffer:	glycerol	50 %
	EDTA (pH 8.0)	1 mM
	Bromophenol blue	0.25 %
	Xylene cyanol FF	0.25 %

Xylene cyanol is a tracker dye and is not absolutely neccessary.

Pre-run gel for 5 min at 5-10 V/cm. The run is completed when the bromophenol blue has migrated approximately 8 cm. If samples have not been pre-stained, stain a portion of the gel (not used for blotting) with 0.5 µg ml^{-1} ethidium bromide in 0.1 M ammonium acetate for 30-40 min and view under UV light. Ethidium bromide is a mutagen, UV light is dangerous, especially to the eyes.

Northern blotting

Standard sodium citrate buffer (20 x SSC)	NaCl	175.3 g
	Sodium citrate	88.2 g
	DW	800 ml

Adjust pH to 7.0 with 1 N NaOH, make up to 1 l and autoclave prior to use.

1. Wash the gel in several changes of DEPC treated DW. Gels that contain > 1% agarose or are more than 0.5 mm thick should instead be soaked 20 min in in 0.05 N NaOH, rinsed and soaked 40 min in 20 x SSC,

2. Fill a large baking dish with 20 x SSC, place a shallow support (glass plates, plexiglass) over the dish,

3. Soak a piece of Whatman 3 mm paper in 20 x SSC and place on the support so that it overlaps into the transfer buffer on both sides of the support. Remove air bubbles,

4. Place the gel on top of the filter and place clingfilm around it to ensure buffer will be transferred through the gel,

5. Place the nylon or nitrocellulose filter (cut to size and wetted in 20 x SSC) on top of the gel (without air bubbles being trapped). Top with 2 pieces of Whatman 3 mm filter paper cut to size,

6. Build a stack of green paper towels, cut just smaller than the 3 mm papers. Place a glassplate on top of the stack and weigh it down with a 500 g weight,

The RNA will be transferred to the membrane by capillary movement of the buffer through the gel and the membrane into the paper towels. Transfer will take 6-18 h. After transfer is complete, the positions of the gel slots can be marked on the membrane with a ball point pen,

7. Rinse the membrane in 6 x SSC for 5 minutes, dry on paper towel for 30 min, place between 2 pieces of 3 mm paper and bake 0.5-2.0 h at 80°C, ideally under vacuum. Nylon membranes can be irradiated at 254 nm at 0.15 J cm^{-2} (when dry) to cross-link the RNA,

8. Strips of nitrocellulose filters and some nylon membranes may be stained with methylene blue: Soak the dry filter in 5% acetic acid for 15 minutes, then in 0.5 M sodium acetate, 0.04% methylene blue for 5-10 minutes. Rinse in water 5-10 min.

Appendix E: Primary Culture of Hyaline Cells in TC-100

TC-100 medium was tried for primary *in vitro* culture for *C. maenas* hyaline cells, but the medium has a pH of ca. 6.2 which is unsuitable as the pH of *C. maenas* plasma is ca. 7.4. Leibovitz' L-15 medium, pepared and modified as recommended by Chen & Kou (1989), is a more suitable medium. To grow cells of marine crustaceans, adjust osmolality to ca. 980 mOsmoles l^{-1} with additional NaCl.

Hyaline cells are unlikely to divide, but are useful for phagocytosis and respiratory bust assay and may support some crab viruses. Because they are easy to harvest under sterile conditions, they are useful models for optimising growth conditions.

Cell separation

(modified from Smith & Söderhäll, 1983):

1. Mix 60% v/v sterile (autoclaved) Percoll™ (Pharmacia) with 40 % sterile 8.5 % NaCl (Note: do not mix solutions prior to autoclaving),

2. Fill ca. 2/3 of sterile high speed centrifuge tubes with Percoll™ mix and centrifuge at 48, 000 *g* 20 min at 4°C to form gradients. Gradients will keep up to 1 week at 4°C,

3. Bleed crabs, one at a time, ca. 2.5 ml per animal, into equal volume of ice-cold MA (Appendix A),

4. Carefully overlay haemolymph-MA mix onto Percoll™-gradient,

5. Centrifuge at 2000 *g* 15 min at 4°C to separate cells,

6. Collect hyaline cells from just underneath the surface with sterile transfer pipette. Avoid the contaminants which accumulate on the surface of the gradient,

7. Transfer cells to fresh tube and add 9 volumes of sterile 3.2% NaCl (w/v), 0.05 M Tris, pH 7.4,

8. Centrifuge 600 *g* 10 min 4°C to sediment the cells. This step removes the Percoll™.

Cell culture

1. Re-suspend separated cells in sterile TC-100, containing 10 % FCS, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, osmolality adjusted with NaCl to ca. 980 mOsmols l^{-1}(ca. 2.5% w/v NaCl),

2. Remove a sample and dilute 1/10 in 0.5% (w/v) eosin Y in sterile tris buffered 3.2% NaCl,

3. Count immediately under impoved Neubauer haemocytometer, count ml^{-1} is number in the central square x dilution x 10^4 (use higher dilutions if necessary); dead cells stain pink,

4. Adjust numbers as required; grow cells at maximum 15°C.

Percentage survival of hyaline cells (duplicate counts from 2 crabs) over 6 days.

Mortality increased rapidly during the period of 24 h-72 h, indicating that the cells may be maintained for short periods, but the effect of pH on cell function has to be considered.

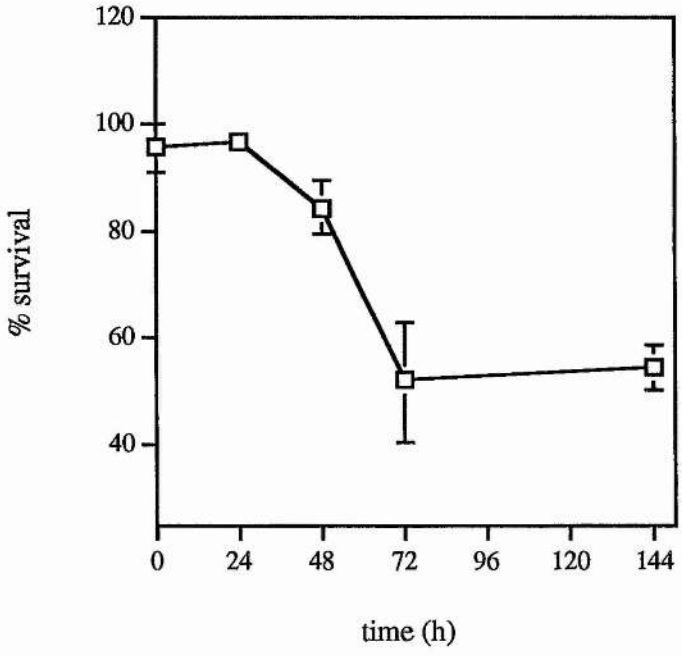

Figure 1. Survival of *C. maenas* hyaline cells in TC 100, prepared as described in the text.